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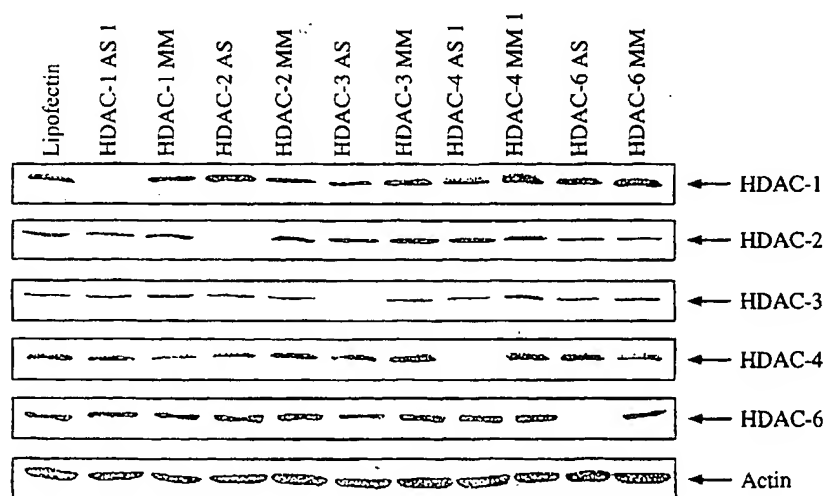
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(54) Title: INHIBITION OF SPECIFIC HISTONE DEACETYLASE ISOFORMS



AS = Antisense
MM = Mismatch
NS = Non-specific control
3 day treatment
Oligonucleotide conc - 50nM

(57) Abstract: This invention relates to the inhibition of histone deacetylase expression and enzymatic activity. The invention provides methods and reagents for inhibiting specific histone deacetylase (HDAC) isoforms by inhibiting expression at the nucleic acid level or enzymatic activity at the protein level.

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INHIBITION OF SPECIFIC HISTONE DEACETYLASE ISOFORMS

5

BACKGROUND OF THE INVENTIONField of the Invention

This invention relates to the fields of inhibition of histone deacetylase expression and enzymatic activity.

10

Summary of the Related Art

In eukaryotic cells, nuclear DNA associates with histones to form a compact complex called chromatin. The histones constitute a family of basic proteins which are generally highly conserved across eukaryotic species. The core histones, termed H2A, H2B, H3, and H4, associate to form a protein core. DNA winds around this protein core, with the basic amino acids of the histones interacting with the negatively charged phosphate groups of the DNA. Approximately 146 base pairs of DNA wrap around a histone core to make up a nucleosome particle, the repeating structural motif of chromatin.

Csordas, *Biochem. J.*, 286: 23-38 (1990) teaches that histones are subject to posttranslational acetylation of the epsilon-amino groups of N-terminal lysine residues, a reaction that is catalyzed by histone acetyltransferase (HAT1). Acetylation neutralizes the positive charge of the lysine side chain, and is thought to impact chromatin structure. Indeed, Taunton *et al.*, *Science*, 272: 408-411 (1996), teaches that access of transcription factors to chromatin templates is enhanced by histone hyperacetylation. Taunton *et al.* further teaches that an enrichment in underacetylated histone H4 has been found in transcriptionally silent regions of the genome.

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Recently, there has been interest in the role of histone deacetylase (HDAC) in gene expression. Sanches Del Pino *et al.*, *Biochem. J.* 303: 723-729 (1994) discloses a partially purified yeast HDAC activity. Taunton *et al.* (*supra*) discloses a human HDAC that is related to a yeast transcriptional
5 regulator and suggests that this protein may be a key regulator of eukaryotic transcription.

Known inhibitors of mammalian HDAC have been used to probe the role of HDAC in gene regulation. Yoshida *et al.*, *J. Biol. Chem.* 265: 17174-17179 (1990) discloses that (R)-Trichostatin A (TSA) is a potent
10 inhibitor of mammalian HDAC. Yoshida *et al.*, *Cancer Research* 47: 3688-3691 (1987) discloses that TSA is a potent inducer of differentiation in murine erythroleukemia cells.

More recently, it has been discovered that the HDAC activity is actually provided by a set of discrete HDAC enzyme isoforms. Grozinger
15 *et al.*, *Proc. Natl. Acad. Sci. (USA)*, 96: 4868-4873 (1999), teaches that HDACs may be divided into two classes, the first represented by yeast Rpd3-like proteins, and the second represented by yeast Hda1-like proteins. Grozinger *et al.* also teaches that the human HDAC1, HDAC2, and HDAC3 proteins are members of the first class of HDACs, and discloses new
20 proteins, named HDAC4, HDAC5, and HDAC6, which are members of the second class of HDACs. Kao *et al.*, *Gene & Development* 14: 55-66 (2000), discloses an additional member of this second class, called HDAC-7. More recently, Hu, E. *et al.*, *J. Bio. Chem.* 275:15254-13264 (2000) disclosed the newest member of the first class of histone deacetylases, HDAC-8. It has
25 been unclear what roles these individual HDAC enzymes play.

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The known inhibitors of histone deacetylase are all small molecules that inhibit histone deacetylase activity at the protein level. Moreover, all of the known histone deacetylase inhibitors are non-specific for a particular histone deacetylase isoform, and more or less inhibit all members of both
5 the histone deacetylase families equally.

Therefore, there remains a need to develop reagents for inhibiting specific histone deacetylase isoforms. There is also a need for the development of methods for using these reagents to identify and inhibit specific histone deacetylase isoforms involved in tumorigenesis.

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BRIEF SUMMARY OF THE INVENTION

The invention provides methods and reagents for inhibiting specific histone deacetylase (HDAC) isoforms by inhibiting expression at the nucleic acid level or enzymatic activity at the protein level. The invention
5 allows the identification of and specific inhibition of specific histone deacetylase isoforms involved in tumorigenesis and thus provides a treatment for cancer. The invention further allows identification of and specific inhibition of specific HDAC isoforms involved in cell proliferation and/or differentiation and thus provides a treatment for cell proliferative
10 and/or differentiation disorders.

The inventors have discovered new agents that inhibit specific HDAC isoforms. Accordingly, in a first aspect, the invention provides agents that inhibit one or more specific histone deacetylase isoforms but less than all histone deacetylase isoforms. Such specific HDAC isoforms
15 include without limitation, HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7 and HDAC-8. Non-limiting examples of the new agents include antisense oligonucleotides (oligos) and small molecule inhibitors specific for one or more HDAC isoforms but less than all HDAC isoforms.

20 The present inventors have surprisingly discovered that specific inhibition of HDAC-1 reverses the tumorigenic state of a transformed cell. The inventors have also surprisingly discovered that the inhibition of the HDAC-4 isoform dramatically induces growth and apoptosis arrest in cancerous cells. Thus, in certain embodiments of this aspect of the
25 invention, the histone deacetylase isoform that is inhibited is HDAC-1 and/or HDAC-4.

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In certain preferred embodiments, the agent that inhibits the specific HDAC isoform is an oligonucleotide that inhibits expression of a nucleic acid molecule encoding that histone deacetylase isoform. The nucleic acid molecule may be genomic DNA (e.g., a gene), cDNA, or RNA. In some
5 embodiments, the oligonucleotide inhibits transcription of mRNA encoding the HDAC isoform. In other embodiments, the oligonucleotide inhibits translation of the histone deacetylase isoform. In certain embodiments the oligonucleotide causes the degradation of the nucleic acid molecule. Particularly preferred embodiments include antisense
10 oligonucleotides directed to HDAC-1 and/or HDAC-4.

In yet other embodiments of the first aspect, the agent that inhibits a specific HDAC isoform is a small molecule inhibitor that inhibits the activity of one or more specific histone deacetylase isoforms but less than all histone deacetylase isoforms.

15 In a second aspect, the invention provides a method for inhibiting one or more, but less than all, histone deacetylase isoforms in a cell, comprising contacting the cell with an agent of the first aspect of the invention. In other preferred embodiments, the agent is an antisense oligonucleotide. In certain preferred embodiments, the agent is a small
20 molecule inhibitor. In other certain preferred embodiments of the second aspect of the invention, cell proliferation is inhibited in the contacted cell. In preferred embodiments, the cell is a neoplastic cell which may be in an animal, including a human, and which may be in a neoplastic growth. In certain preferred embodiments, the method of the second aspect of the
25 invention further comprises contacting the cell with a histone deacetylase small molecule inhibitor that interacts with and reduces the enzymatic activity of one or more specific histone deacetylase isoforms. In still yet other preferred embodiments of the second aspect of the invention, the method comprises an agent of the first aspect of the invention which is a

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combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In
5 other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4. In some embodiments, the histone deacetylase small molecule inhibitor is operably associated with the antisense oligonucleotide.

In a third aspect, the invention provides a method for inhibiting
10 neoplastic cell proliferation in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of an agent of the first aspect of the invention. In certain preferred embodiments, the agent is an antisense oligonucleotide which is combined with a pharmaceutically acceptable
15 carrier and administered for a therapeutically effective period of time. In certain preferred embodiments, the agent is a small molecule inhibitor which is combined with a pharmaceutically acceptable carrier and administered for a therapeutically effective period of time. In certain preferred embodiments of the this aspect of the invention, cell proliferation
20 is inhibited in the contacted cell. In preferred embodiments, the cell is a neoplastic cell which may be in an animal, including a human, and which may be in a neoplastic growth. In other certain embodiments, the agent is a small molecule inhibitor of the first aspect of the invention which is combined with a pharmaceutically acceptable carrier and administered for
25 a therapeutically effective period of time. In still yet other preferred embodiments of the third aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred

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embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

- 5 In a fourth aspect, the invention provides a method for identifying a specific histone deacetylase isoform that is required for induction of cell proliferation comprising contacting a cell with an agent of the first aspect of the invention. In certain preferred embodiments, the agent is an antisense oligonucleotide that inhibits the expression of a histone
- 10 deacetylase isoform, wherein the antisense oligonucleotide is specific for a particular HDAC isoform, and thus inhibition of cell proliferation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is required for induction of cell proliferation. In other certain embodiments, the agent is a small molecule inhibitor that
- 15 inhibits the activity of a histone deacetylase isoform, wherein the small molecule inhibitor is specific for a particular HDAC isoform, and thus inhibition of cell proliferation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is required for induction of cell proliferation. In certain preferred embodiments, the cell is
- 20 a neoplastic cell, and the induction of cell proliferation is tumorigenesis. In still yet other preferred embodiments of the fourth aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In
- 25 certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

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In an fifth aspect, the invention provides a method for identifying a histone deacetylase isoform that is involved in induction of cell differentiation, comprising contacting a cell with an agent that inhibits the expression of a histone deacetylase isoform, wherein induction of

5 differentiation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is involved in induction of cell differentiation. In certain preferred embodiments, the agent is an antisense oligonucleotide of the first aspect of the invention. In other certain preferred embodiments, the agent is a small molecule inhibitor of

10 the first aspect of the invention. In still other certain embodiments, the cell is a neoplastic cell. In still yet other preferred embodiments of the fifth aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first

15 aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In a sixth aspect, the invention provides a method for inhibiting

20 neoplastic cell growth in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of an agent of the first aspect of the invention. In certain embodiments thereof, the agent is an antisense oligonucleotide, which is combined with a pharmaceutically acceptable carrier and administered for

25 a therapeutically effective period of time.

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In an seventh aspect, the invention provides a method for identifying a histone deacetylase isoform that is involved in induction of cell differentiation, comprising contacting a cell with an antisense oligonucleotide that inhibits the expression of a histone deacetylase isoform, wherein induction of differentiation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is involved in induction of cell differentiation. Preferably, the cell is a neoplastic cell. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In an eighth aspect, the invention provides a method for inhibiting cell proliferation in a cell comprising contacting a cell with at least two reagents selected from the group consisting of an antisense oligonucleotide from the first aspect of the invention that inhibits expression of a specific histone deacetylase isoform, a small molecule inhibitor from the first aspect of the invention that inhibits a specific histone deacetylase isoform, an antisense oligonucleotide that inhibits a DNA methyltransferase, and a small molecule that inhibits a DNA methyltransferase. In one embodiment, the inhibition of cell growth of the contacted cell is greater than the inhibition of cell growth of a cell contacted with only one of the reagents. In certain embodiments, each of the reagents selected from the group is substantially pure. In preferred embodiments, the cell is a neoplastic cell. In yet additional preferred embodiments, the reagents selected from the group are operably associated. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

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In a ninth aspect, the invention provides a method for modulating cell proliferation or differentiation, comprising contacting a cell with an agent of the first aspect of the invention, wherein one or more, but less than all, HDAC isoforms are inhibited, which results in a modulation of

5 proliferation or differentiation. In certain embodiments, the agent is an antisense oligonucleotide of the first aspect of the invention. In other certain preferred embodiments, the agent is a small molecule inhibitor of the first aspect of the invention. In preferred embodiments, the cell proliferation is neoplasia. In still yet other preferred embodiments of the

10 this aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5,

15 HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a schematic diagram providing the amino acid sequence of HDAC-1, as provided in GenBank Accession No. AAC50475 (SEQ ID NO:1).

5

Figure 1B is a schematic diagram providing the nucleic acid sequence of HDAC-1, as provided in GenBank Accession No. U50079 (SEQ ID NO:2).

10 Figure 2A is a schematic diagram providing the amino acid sequence of HDAC-2, as provided in GenBank Accession No. AAC50814 (SEQ ID NO:3).

15 Figure 2B is a schematic diagram providing the nucleic acid sequence of HDAC-2, as provided in GenBank Accession No. U31814 (SEQ ID NO:4).

20 Figure 3A is a schematic diagram providing the amino acid sequence of HDAC-3, as provided in GenBank Accession No. AAB88241 (SEQ ID NO:5).

25 Figure 3B is a schematic diagram providing the nucleic acid sequence of HDAC-3, as provided in GenBank Accession No. U75697 (SEQ ID NO:6).

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Figure 4A is a schematic diagram providing the amino acid sequence of HDAC-4, as provided in GenBank Accession No. BAA22957 (SEQ ID NO:7).

5 Figure 4B is a schematic diagram providing the nucleic acid sequence of HDAC-4, as provided in GenBank Accession No. AB006626 (SEQ ID NO:8).

10 Figure 5A is a schematic diagram providing the amino acid sequence of HDAC-5, as provided in GenBank Accession No. BAA25526 (SEQ ID NO:9).

15 Figure 5B is a schematic diagram providing the nucleic acid sequence of HDAC-5 as provided in GenBank Accession No. AB011172 (SEQ ID NO:10).

20 Figure 6A is a schematic diagram providing the amino acid sequence of human HDAC-6, as provided in GenBank Accession No. AAD29048 (SEQ ID NO:11).

Figure 6B is a schematic diagram providing the nucleic acid sequence of human HDAC-6, as provided in GenBank Accession No. AJ011972 (SEQ ID NO:12).

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Figure 7A is a schematic diagram providing the amino acid sequence of human HDAC-7, as provided in GenBank Accession No. AAF63491.1 (SEQ ID NO:13).

5 Figure 7B is a schematic diagram providing the nucleic acid sequence of human HDAC-7, as provided in GenBank Accession No. AF239243 (SEQ ID NO:14).

10 Figure 8A is a schematic diagram providing the amino acid sequence of human HDAC-8, as provided in GenBank Accession No. AAF73076.1 (SEQ ID NO:15).

15 Figure 8B is a schematic diagram providing the nucleic acid sequence of human HDAC-8, as provided in GenBank Accession No. AF230097 (SEQ ID NO:16).

20 Figure 9A is a representation of a Northern blot demonstrating the effect of HDAC-1 AS1 antisense oligonucleotide on HDAC-1 mRNA expression in human A549 cells.

25 Figure 9A is a representation of a Northern blot demonstrating the effect of HDAC-2 AS antisense oligonucleotide on HDAC-2 mRNA expression in human A549 cells.

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Figure 9C is a representation of a Northern blot demonstrating the effect of HDAC-6 AS antisense oligonucleotide on HDAC-6 mRNA expression in human A549 cells.

5 Figure 9D is a representation of a Northern blot demonstrating the effect of HDAC-3 AS antisense oligonucleotide on HDAC-3 mRNA expression in human A549 cells.

10 Figure 9E is a representation of a Northern blot demonstrating the effect of an HDAC-4 antisense oligonucleotide (AS1) on HDAC-4 mRNA expression in human A549 cells.

15 Figure 9F is a representation of a Northern blot demonstrating the dose-dependent effect of an HDAC-4 antisense oligonucleotide (AS2) on HDAC-4 mRNA expression in human A549 cells.

20 Figure 9G is a representation of a Northern blot demonstrating the effect of an HDAC-5 antisense oligonucleotide (AS) on HDAC-5 mRNA expression in human A549 cells.

Figure 9H is a representation of a Northern blot demonstrating the effect of an HDAC-7 antisense oligonucleotide (AS) on HDAC-7 mRNA expression in human A549 cells.

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Figure 9I is a representation of a Northern blot demonstrating the dose-dependent effect of HDAC-8 antisense oligonucleotides (AS1 and AS2) on HDAC-8 mRNA expression in human A549 cells.

5 Figure 10A is a representation of a Western blot demonstrating the effect of HDAC isotype-specific antisense oligos on HDAC isotype protein expression in human A549 cells.

10 Figure 10B is a representation of a Western blot demonstrating the dose-dependent effect of the HDAC-1 isotype-specific antisense oligo (AS1 and AS2) on HDAC isotype protein expression in human A549 cells.

15 Figure 10C is a representation of a Western blot demonstrating the effect of HDAC-4 isotype-specific antisense oligonucleotide (AS2) on HDAC isotype protein expression in human A549 cells.

20 Figure 11A is a graphic representation demonstrating the apoptotic effect of HDAC isotype-specific antisense oligos on human A549 cancer cells.

 Figure 12A is a graphic representation demonstrating the effect of HDAC-1 AS1 and AS2 antisense oligonucleotides on the proliferation of human A549 cancer cells.

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Figure 12B is a graphic representation demonstrating the effect of HDAC-8 specific AS1 and AS2 antisense oligonucleotides on the proliferation of human A549 cancer cells.

5 Figure 13 is a a graphic representation demonstrating the cell cycle blocking effect of HDAC specific antisense oligonucleotides on human A549 cancer cells.

Figure 14 is a representation of an RNase protection assay
10 demonstrating the effect of HDAC isotype-specific antisense oligonucleotides on HDAC isotype mRNA expression in human A549 cells.

Figure 15 is a representation of a Western blot demonstrating that treatment of human A549 cells with HDAC-4 AS1 antisense
15 oligonucleotide induces the expression of the p21 protein.

Figure 16 is a representation of a Western blot demonstrating that treatment of human A549 cells with HDAC-1 antisense oligonucleotides (AS1 and AS2) represses the expression of the cyclin B1 and cyclin A genes.
20

Figure 17 shows plating data demonstrating the ability of antisense oligonucleotides complementary to HDAC-1 to inhibit growth in soft agar of A549 cells far more than can antisense oligonucleotides complementary to HDAC-2, HDAC-6 or mismatched controls.

25

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Figure 18 is a representation of a Western blot demonstrating that treatment of human A549 cells with the small molecule inhibitor Compound 3 (Table 2) induces the expression of the p21 protein and represses the expression of the cyclin B1 and cyclin A genes.

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DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention provides methods and reagents for inhibiting specific histone deacetylase isoforms (HDAC) by inhibiting expression at the nucleic acid level or protein activity at the enzymatic level. The invention
5 allows the identification of and specific inhibition of specific histone deacetylase isoforms involved in tumorigenesis and thus provides a treatment for cancer. The invention further allows identification of and specific inhibition of specific HDAC isoforms involved in cell proliferation and/or differentiation and thus provides a treatment for cell proliferative
10 and/or differentiation disorders.

The patent and scientific literature referred to herein establishes knowledge that is available to those with skill in the art. The issued patents, applications, and references, including GenBank database sequences, that are cited herein are hereby incorporated by reference to the
15 same extent as if each was specifically and individually indicated to be incorporated by reference.

In a first aspect, the invention provides agents that inhibit one or more histone deacetylase isoform, but less than all specific histone deacetylase isoforms. As used herein interchangeably, the terms "histone deacetylase", "HDAC", "histone deacetylase isoform", "HDAC isoform" and
20 similar terms are intended to refer to any one of a family of enzymes that remove acetyl groups from the epsilon-amino groups of lysine residues at the N-terminus of a histone. Unless otherwise indicated by context, the term "histone" is meant to refer to any histone protein, including H1, H2A, H2B, H3, and H4, from any species. Preferred histone deacetylase isoforms include class I and class II enzymes. Specific HDACs include without
25 limitation, HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7 and HDAC-8. By way of non-limiting example, useful agents that

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inhibit one or more histone deacetylase isoforms, but less than all specific histone deacetylase isoforms, include antisense oligonucleotides and small molecule inhibitors.

The present inventors have surprisingly discovered that specific inhibition of HDAC-1 reverses the tumorigenic state of a transformed cell. The inventors have also surprisingly discovered that the inhibition of the HDAC-4 isoform dramatically induces growth and apoptosis arrest in cancerous cells. Thus, in certain embodiments of this aspect of the invention, the histone deacetylase isoform that is inhibited is HDAC-1 and/or HDAC-4.

Preferred agents that inhibit HDAC-1 and/or HDAC-4 dramatically inhibit growth of human cancer cells, independent of p53 status. These agents significantly induce apoptosis in the cancer cells and cause dramatic growth arrest. They also can induce transcription of tumor suppressor genes, such as p21^{WAF1}, p57^{KIP2}, GADD153 and GADD45. Finally, they exhibit both *in vitro* and *in vivo* anti-tumor activity. Inhibitory agents that achieve one or more of these results are considered within the scope of this aspect of the invention. By way of non-limiting example, antisense oligonucleotides and/or small molecule inhibitors of HDAC-1 and/or HDAC-4 are useful for the invention.

In certain preferred embodiments, the agent that inhibits the specific HDAC isoform is an oligonucleotide that inhibits expression of a nucleic acid molecule encoding a specific histone deacetylase isoform. The nucleic acid molecule may be genomic DNA (*e.g.*, a gene), cDNA, or RNA. In other embodiments, the oligonucleotide ultimately inhibits translation of the histone deacetylase. In certain embodiments the oligonucleotide causes the degradation of the nucleic acid molecule. Preferred antisense

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oligonucleotides have potent and specific antisense activity at nanomolar concentrations.

The antisense oligonucleotides according to the invention are complementary to a region of RNA or double-stranded DNA that encodes
5 a portion of one or more histone deacetylase isoform (taking into account that homology between different isoforms may allow a single antisense oligonucleotide to be complementary to a portion of more than one isoform).

For purposes of the invention, the term "complementary" means
10 having the ability to hybridize to a genomic region, a gene, or an RNA transcript thereof under physiological conditions. Such hybridization is ordinarily the result of base-specific hydrogen bonding between complementary strands, preferably to form Watson-Crick or Hoogsteen base pairs, although other modes of hydrogen bonding, as well as base
15 stacking can lead to hybridization. As a practical matter, such hybridization can be inferred from the observation of specific gene expression inhibition, which may be at the level of transcription or translation (or both).

For purposes of the invention, the term "oligonucleotide" includes
20 polymers of two or more deoxyribonucleosides, ribonucleosides, or 2'-O-substituted ribonucleoside residues, or any combination thereof. Preferably, such oligonucleotides have from about 8 to about 50 nucleoside residues, and most preferably from about 12 to about 30 nucleoside residues. The nucleoside residues may be coupled to each other by any of
25 the numerous known internucleoside linkages. Such internucleoside linkages include without limitation phosphorothioate, phosphorodithioate, alkylphosphonate, alkylphosphonothioate, phosphotriester, phosphoramidate, siloxane, carbonate, carboxymethylester, acetamidate,

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carbamate, thioether, bridged phosphoramidate, bridged methylene
phosphonate, bridged phosphorothioate, and sulfone internucleotide
linkages. In certain preferred embodiments, these internucleoside linkages
may be phosphodiester, phosphotriester, phosphorothioate, or
5 phosphoramidate linkages, or combinations thereof. The term
oligonucleotide also encompasses such polymers having chemically
modified bases or sugars and/or having additional substituents, including
without limitation lipophilic groups, intercalating agents, diamines, and
adamantane. The term oligonucleotide also encompasses such polymers as
10 PNA and LNA. For purposes of the invention the term "2'-O-substituted"
means substitution of the 2' position of the pentose moiety with an -O-
lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or
with an -O-aryl or allyl group having 2-6 carbon atoms, wherein such alkyl,
aryl, or allyl group may be unsubstituted or may be substituted, *e.g.*, with
15 halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy,
carboxyl, carbalkoxyl, or amino groups; or such 2' substitution may be with
a hydroxy group (to produce a ribonucleoside), an amino or a halo group,
but not with a 2'-H group.

Particularly preferred antisense oligonucleotides utilized in this
20 aspect of the invention include chimeric oligonucleotides and hybrid
oligonucleotides.

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For purposes of the invention, a "chimeric oligonucleotide" refers to an oligonucleotide having more than one type of internucleoside linkage. One preferred embodiment of such a chimeric oligonucleotide is a chimeric oligonucleotide comprising a phosphorothioate, phosphodiester or
5 phosphorodithioate region, preferably comprising from about 2 to about 12 nucleotides, and an alkylphosphonate or alkylphosphonothioate region (see *e.g.*, Pederson *et al.* U.S. Patent Nos. 5,635,377 and 5,366,878). Preferably, such chimeric oligonucleotides contain at least three consecutive internucleoside linkages selected from phosphodiester and
10 phosphorothioate linkages, or combinations thereof.

For purposes of the invention, a "hybrid oligonucleotide" refers to an oligonucleotide having more than one type of nucleoside. One preferred embodiment of such a hybrid oligonucleotide comprises a ribonucleotide or 2'-O-substituted ribonucleotide region, preferably
15 comprising from about 2 to about 12 2'-O-substituted nucleotides, and a deoxyribonucleotide region. Preferably, such a hybrid oligonucleotide will contain at least three consecutive deoxyribonucleosides and will also contain ribonucleosides, 2'-O-substituted ribonucleosides, or combinations thereof (see *e.g.*, Metelev and Agrawal, U.S. Patents Nos. 5,652,355 and
20 5,652,356).

The exact nucleotide sequence and chemical structure of an antisense oligonucleotide utilized in the invention can be varied, so long as the oligonucleotide retains its ability to inhibit expression of a specific histone deacetylase isoform or inhibit one or more histone deacetylase
25 isoforms, but less than all specific histone deacetylase isoforms. This is readily determined by testing whether the particular antisense oligonucleotide is active by quantitating the amount of mRNA encoding a specific histone deacetylase isoform, quantitating the amount of histone

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deacetylase isoform protein, quantitating the histone deacetylase isoform enzymatic activity, or quantitating the ability of the histone deacetylase isoform to inhibit cell growth in a an *in vitro* or *in vivo* cell growth assay, all of which are described in detail in this specification. The term "inhibit
5 expression" and similar terms used herein are intended to encompass any one or more of these parameters.

Antisense oligonucleotides utilized in the invention may conveniently be synthesized on a suitable solid support using well-known chemical approaches, including H-phosphonate chemistry,
10 phosphoramidite chemistry, or a combination of H-phosphonate chemistry and phosphoramidite chemistry (*i.e.*, H-phosphonate chemistry for some cycles and phosphoramidite chemistry for other cycles). Suitable solid supports include any of the standard solid supports used for solid phase oligonucleotide synthesis, such as controlled-pore glass (CPG) (see, *e.g.*,
15 Pon, R. T., Methods in Molec. Biol. 20: 465-496, 1993).

Antisense oligonucleotides according to the invention are useful for a variety of purposes. For example, they can be used as "probes" of the physiological function of specific histone deacetylase isoforms by being used to inhibit the activity of specific histone deacetylase isoforms in an
20 experimental cell culture or animal system and to evaluate the effect of inhibiting such specific histone deacetylase isoform activity. This is accomplished by administering to a cell or an animal an antisense oligonucleotide that inhibits one or more histone deacetylase isoform expression according to the invention and observing any phenotypic
25 effects. In this use, the antisense oligonucleotides according to the invention is preferable to traditional "gene knockout" approaches because it is easier to use, and can be used to inhibit specific histone deacetylase isoform activity at selected stages of development or differentiation.

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- Preferred antisense oligonucleotides of the invention inhibit either the transcription of a nucleic acid molecule encoding the histone deacetylase isoform, and/or the translation of a nucleic acid molecule encoding the histone deacetylase isoform, and/or lead to the degradation of such nucleic acid. Histone deacetylase-encoding nucleic acids may be RNA or double stranded DNA regions and include, without limitation, intronic sequences, untranslated 5' and 3' regions, intron-exon boundaries as well as coding sequences from a histone deacetylase family member gene. For human sequences, see *e.g.*, Yang et al., *Proc. Natl. Acad. Sci. (USA)* 93(23): 12845-12850, 1996; Furukawa et al., *Cytogenet. Cell Genet.* 73(1-2): 130-133, 1996; Yang et al., *J. Biol. Chem.* 272(44): 28001-28007, 1997; Betz et al., *Genomics* 52(2): 245-246, 1998; Taunton et al., *Science* 272(5260): 408-411, 1996; and Dangond et al., *Biochem. Biophys. Res. Commun.* 242(3): 648-652, 1998).
- Particularly preferred non-limiting examples of antisense oligonucleotides of the invention are complementary to regions of RNA or double-stranded DNA encoding a histone deacetylase isoform (*e.g.*, HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8). (see *e.g.*, GenBank Accession No. U50079 for human HDAC-1 (Fig. 1B); GenBank Accession No. U31814 for human HDAC-2; (Fig. 2B) GenBank Accession No. U75697 for human HDAC-3 (Fig. 3B; GenBank Accession No. AB006626 for human HDAC-4 (Fig. 4B); GenBank Accession No. AB011172 for human HDAC-5 (Fig. 5B); GenBank Accession No. AJ011972 for human HDAC-6 (Fig. 6B); GenBank Accession No. AF239243 for human HDAC-7 (Fig. 7B); and GenBank Accession No. AF230097 for human HDAC-8 (Fig. 8B)).

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The sequences encoding histone deacetylases from many non-human animal species are also known (see, for example, GenBank Accession Numbers X98207 (murine HDAC-1); NM_008229 (murine HDAC-2); NM_010411 (murine HDAC-3); NM_006037 (murine HDAC-4);
5 NM_010412 (murine HDAC-5); NM_010413 (murine HDAC-6); and AF207749 (murine HDAC-7)). Accordingly, the antisense oligonucleotides of the invention may also be complementary to regions of RNA or double-stranded DNA that encode histone deacetylases from non-human animals. Antisense oligonucleotides according to these embodiments are useful as
10 tools in animal models for studying the role of specific histone deacetylase isoforms.

Particularly, preferred oligonucleotides have nucleotide sequences of from about 13 to about 35 nucleotides which include the nucleotide sequences shown in Table I. Yet additional particularly preferred
15 oligonucleotides have nucleotide sequences of from about 15 to about 26 nucleotides of the nucleotide sequences shown below. Most preferably, the oligonucleotides shown below have phosphorothioate backbones, are 20-26 nucleotides in length, and are modified such that the terminal four nucleotides at the 5' end of the oligonucleotide and the terminal four
20 nucleotides at the 3' end of the oligonucleotide each have 2'-O- methyl groups attached to their sugar residues.

Antisense oligonucleotides used in the present study are shown in Table I.

Table 1
Sequences of Human Isotype-Specific Antisense (AS)
Oligonucleotides and Their Mismatch (MM) Oligonucleotides

Oligo	Target	Accession Number	Nucleotide Position	Sequence	Gene Position
HDAC1 AS1	Human HDAC1	U50079	1585-1604	5'-GAAACGTGAGGGACTCAGCA-3' (SEQ ID NO:17)	3'-UTR
HDAC1 AS2	Human HDAC1	U50079	1565-1584	5'-GGAAGCCAGAGCTGGAGAAG-3' (SEQ ID NO:18)	3'-UTR
HDAC1 MM	Human HDAC1	U50079	1585-1604	5'-GTTAGGTGAGGCACTGAGGA-3' (SEQ ID NO:19)	3'-UTR
HDAC2 AS	Human HDAC2	U31814	1643-1622	5'-GCTGAGCTGTTCTGATTGG-3' (SEQ ID NO:20)	3'-UTR
HDAC2 MM	Human HDAC2	U31814	1643-1622	5'-CGTGAGCACTTCTCATTCC-3' (SEQ ID NO:21)	3'-UTR
HDAC3 AS	Human HDAC3	AF039703	1276-1295	5'-CGCTTTCCTTGTCATTGACA-3' (SEQ ID NO:22)	3'-UTR
HDAC3 MM	Human HDAC3	AF039703	1276-1295	5'-GCCTTTCCTACTCATTTGT-3' (SEQ ID NO:23)	3'-UTR
HDAC4 AS1	Human HDAC4	AB006626	514-33	5'-GCTGCCTGCCGTGCCACCC-3' (SEQ ID NO:24)	5'-UTR
HDAC4 MM1	Human HDAC4	AB006626	514-33	5'-CGTGCCTGCGCTGCCACGG-3' (SEQ ID NO:25)	5'-UTR
HDAC4 AS2	Human HDAC4	AB006626	7710-29	5'-TACAGTCCATGCAACCTCCA-3' (SEQ ID NO:26)	3'-UTR
HDAC4 MM4	Human HDAC4	AB006626	7710-29	5'-ATCAGTCCAACCAACCTCGT-3' (SEQ ID NO:27)	3'-UTR
HDAC5 AS	Human HDAC5	AF039691	2663-2682	5'-CTTCGGTCTCACCTGCTTGG-3' (SEQ ID NO:28)	3'-UTR
HDAC6 AS	Human HDAC6	AJ011972	3791-3810	5'-CAGGCTGGAATGAGCTACAG-3' (SEQ ID NO:29)	3'-UTR
HDAC6 MM	Human HDAC6	AJ011972	3791-3810	5'-GACGCTGCAATCAGGTAGAC-3' (SEQ ID NO:30)	3'-UTR
HDAC7 AS	Human HDAC7	AF239243	2896-2915	5'-CTTCAGCCAGGATGCCACA-3' (SEQ ID NO:31)	3'-UTR
HDAC8 AS1	Human HDAC8	AF230097	51-70	5'-CTCCGGCTCCTCCATCTTC-3' (SEQ ID NO:32)	5'-UTR
HDAC8 AS2	Human HDAC8	AF230097	1328-1347	5'-AGCCAGCTGCCACTTGATGC-3' (SEQ ID NO:33)	3'-UTR

The antisense oligonucleotides according to the invention may optionally be formulated with any of the well known pharmaceutically acceptable carriers or diluents (see preparation of pharmaceutically acceptable formulations in, *e.g.*, Remington's Pharmaceutical Sciences, 18th Edition, ed. A. Gennaro, Mack Publishing Co., Easton, PA, 1990), with the proviso that such carriers or diluents not affect their ability to modulate HDAC activity.

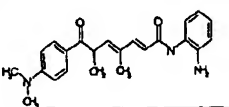
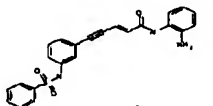
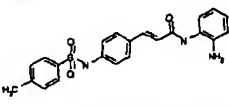
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By way of non-limiting example, the agent of the first aspect of the invention may also be a small molecule inhibitor. The term "small molecule" as used in reference to the inhibition of histone deacetylase is used to identify a compound having a molecular weight preferably less than 1000 Da, more preferably less than 800 Da, and most preferably less than 600 Da, which is capable of interacting with a histone deacetylase and inhibiting the expression of a nucleic acid molecule encoding an HDAC isoform or activity of an HDAC protein. Inhibiting histone deacetylase enzymatic activity means reducing the ability of a histone deacetylase to remove an acetyl group from a histone. In some preferred embodiments, such reduction of histone deacetylase activity is at least about 50%, more preferably at least about 75%, and still more preferably at least about 90%. In other preferred embodiments, histone deacetylase activity is reduced by at least 95% and more preferably by at least 99%. In one certain embodiment, the small molecule inhibitor is an inhibitor of one or more but less than all HDAC isoforms. By "all HDAC isoforms" is meant all proteins that specifically remove an epsilon acetyl group from an N-terminal lysine of a histone, and includes, without limitation, HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8, all of which are considered "related proteins," as used herein.

Most preferably, a histone deacetylase small molecule inhibitor interacts with and reduces the activity of one or more histone deacetylase isoforms (*e.g.*, HDAC-1 and/or HDAC-4), but does not interact with or reduce the activities of all of the other histone deacetylase isoforms (*e.g.*, HDAC-2 and HDAC-6). As discussed below, a preferred histone deacetylase small molecule inhibitor is one that interacts with and reduces the enzymatic activity of a histone deacetylase isoform that is involved in tumorigenesis.

Non-limiting examples of small molecule inhibitors useful for the invention are presented in Table 2.

Table 2

Small Molecule HDAC Inhibitors [μ M] and Their Antitumor Activities <i>In Vivo</i>												
Cpd	Inhibitor Structure	Enzyme IC ₅₀ (μ M)						% Inhibitor of tumor formation in vivo				
		HDAC1	HDAC2	HDAC3	HDAC4	HDAC6	H4-Ac	MIT	Cell Cycle Arrest EC	colon	lung	prostate
1		3	25	21	23	>50	1	3	2			
2		3	31	30	35	>30	5	4	8	53 (40,po)	54 (50,ip)	
3		3	22	45	28	>50	5	4	2	55 (40,ip)		
note: for <i>in vivo</i> antitumor studies, numbers outside brackets indicate % of inhibition of tumor growth in vivo; numbers in brackets indicate daily dose of inhibitor used (mg/kg body weight/day); oral (PO) or intraperitoneal (IP) administration is indicated in brackets.												

5 The reagents according to the invention are useful as analytical tools and as therapeutic tools, including as gene therapy tools. The invention also provides methods and compositions which may be manipulated and fine-tuned to fit the condition(s) to be treated while producing fewer side effects.

10 In a second aspect, the invention provides a method for inhibiting one or more, but less than all, histone deacetylase isoforms in a cell comprising contacting the cell with an agent of the first aspect of the invention. By way of non-limiting example, the agent may be an antisense oligonucleotide or a small molecule inhibitor that inhibits the expression of

15 one or more, but less than all, specific histone deacetylase isoforms in the cell.

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In one certain embodiment, the invention provides a method comprising contacting a cell with an antisense oligonucleotide that inhibits one or more but less than all histone deacetylase isoforms in the cell. Preferably, cell proliferation is inhibited in the contacted cell. Thus, the antisense oligonucleotides according to the invention are useful in therapeutic approaches to human diseases including benign and malignant neoplasms by inhibiting cell proliferation in cells contacted with the antisense oligonucleotides. The phrase "inhibiting cell proliferation" is used to denote an ability of a histone deacetylase antisense oligonucleotide or a small molecule histone deacetylase inhibitor (or combination thereof) to retard the growth of cells contacted with the oligonucleotide or small molecule inhibitor, as compared to cells not contacted. Such an assessment of cell proliferation can be made by counting contacted and non-contacted cells using a Coulter Cell Counter (Coulter, Miami, FL) or a hemacytometer. Where the cells are in a solid growth (*e.g.*, a solid tumor or organ), such an assessment of cell proliferation can be made by measuring the growth with calipers, and comparing the size of the growth of contacted cells with non-contacted cells. Preferably, the term includes a retardation of cell proliferation that is at least 50% of non-contacted cells. More preferably, the term includes a retardation of cell proliferation that is 100% of non-contacted cells (*i.e.*, the contacted cells do not increase in number or size). Most preferably, the term includes a reduction in the number or size of contacted cells, as compared to non-contacted cells. Thus, a histone deacetylase antisense oligonucleotide or a histone deacetylase small molecule inhibitor that inhibits cell proliferation in a contacted cell may induce the contacted cell to undergo growth retardation, to undergo growth arrest, to undergo programmed cell death (*i.e.*, to apoptose), or to undergo necrotic cell death.

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Conversely, the phrase "inducing cell proliferation" and similar terms are used to denote the requirement of the presence or enzymatic activity of a specific histone deacetylase isoform for cell proliferation in a normal (*i.e.*, non-neoplastic) cell. Hence, over-expression of a specific
5 histone deacetylase isoform that induces cell proliferation may or may not lead to increased cell proliferation; however, inhibition of a specific histone deacetylase isoform that induces cell proliferation will lead to inhibition of cell proliferation.

The cell proliferation inhibiting ability of the antisense
10 oligonucleotides according to the invention allows the synchronization of a population of a-synchronously growing cells. For example, the antisense oligonucleotides of the invention may be used to arrest a population of non-neoplastic cells grown *in vitro* in the G1 or G2 phase of the cell cycle. Such synchronization allows, for example, the identification of gene
15 and/or gene products expressed during the G1 or G2 phase of the cell cycle. Such a synchronization of cultured cells may also be useful for testing the efficacy of a new transfection protocol, where transfection efficiency varies and is dependent upon the particular cell cycle phase of the cell to be transfected. Use of the antisense oligonucleotides of the
20 invention allows the synchronization of a population of cells, thereby aiding detection of enhanced transfection efficiency.

The anti-neoplastic utility of the antisense oligonucleotides according to the invention is described in detail elsewhere in this specification.

25 In yet other preferred embodiments, the cell contacted with a histone deacetylase antisense oligonucleotide is also contacted with a histone deacetylase small molecule inhibitor.

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In a few preferred embodiments, the histone deacetylase small molecule inhibitor is operably associated with the antisense oligonucleotide. As mentioned above, the antisense oligonucleotides according to the invention may optionally be formulated well known
5 pharmaceutically acceptable carriers or diluents. This formulation may further contain one or more one or more additional histone deacetylase antisense oligonucleotide(s), and/or one or more histone deacetylase small molecule inhibitor(s), or it may contain any other pharmacologically active agent.

10 In a particularly preferred embodiment of the invention, the antisense oligonucleotide is in operable association with a histone deacetylase small molecule inhibitor. The term "operable association" includes any association between the antisense oligonucleotide and the histone deacetylase small molecule inhibitor which allows an antisense
15 oligonucleotide to inhibit one or more specific histone deacetylase isoform-encoding nucleic acid expression and allows the histone deacetylase small molecule inhibitor to inhibit specific histone deacetylase isoform enzymatic activity. One or more antisense oligonucleotide of the invention may be operably associated with one or more histone deacetylase small molecule
20 inhibitor. In some preferred embodiments, an antisense oligonucleotide of the invention that targets one particular histone deacetylase isoform (e.g., HDAC-1) is operably associated with a histone deacetylase small molecule inhibitor which targets the same histone deacetylase isoform. A preferred operable association is a hydrolyzable. Preferably, the hydrolyzable
25 association is a covalent linkage between the antisense oligonucleotide and the histone deacetylase small molecule inhibitor. Preferably, such covalent linkage is hydrolyzable by esterases and/or amidases. Examples of such hydrolyzable associations are well known in the art. Phosphate esters are particularly preferred.

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In certain preferred embodiments, the covalent linkage may be directly between the antisense oligonucleotide and the histone deacetylase small molecule inhibitor so as to integrate the histone deacetylase small molecule inhibitor into the backbone. Alternatively, the covalent linkage
5 may be through an extended structure and may be formed by covalently linking the antisense oligonucleotide to the histone deacetylase small molecule inhibitor through coupling of both the antisense oligonucleotide and the histone deacetylase small molecule inhibitor to a carrier molecule such as a carbohydrate, a peptide or a lipid or a glycolipid. Other
10 preferred operable associations include lipophilic association, such as formation of a liposome containing an antisense oligonucleotide and the histone deacetylase small molecule inhibitor covalently linked to a lipophilic molecule and thus associated with the liposome. Such lipophilic molecules include without limitation phosphatidylcholine, cholesterol,
15 phosphatidylethanolamine, and synthetic neoglycolipids, such as syallylacNAc-HDPE. In certain preferred embodiments, the operable association may not be a physical association, but simply a simultaneous existence in the body, for example, when the antisense oligonucleotide is associated with one liposome and the small molecule inhibitor is associated
20 with another liposome.

In a third aspect, the invention provides a method for inhibiting neoplastic cell proliferation in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of an agent of the first aspect of the
25 invention. In one certain embodiment, the agent is an antisense oligonucleotide of the first aspect of the invention, and the method further comprises a pharmaceutically acceptable carrier. The antisense oligonucleotide and the pharmaceutically acceptable carrier are administered for a therapeutically effective period of time. Preferably, the

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animal is a mammal, particularly a domesticated mammal. Most preferably, the animal is a human.

The term "neoplastic cell" is used to denote a cell that shows aberrant cell growth. Preferably, the aberrant cell growth of a neoplastic cell is increased cell growth. A neoplastic cell may be a hyperplastic cell, a cell that shows a lack of contact inhibition of growth *in vitro*, a benign tumor cell that is incapable of metastasis *in vivo*, or a cancer cell that is capable of metastases *in vivo* and that may recur after attempted removal. The term "tumorigenesis" is used to denote the induction of cell proliferation that leads to the development of a neoplastic growth.

The terms "therapeutically effective amount" and "therapeutically effective period of time" are used to denote known treatments at dosages and for periods of time effective to reduce neoplastic cell growth. Preferably, such administration should be parenteral, oral, sublingual, transdermal, topical, intranasal, or intrarectal. When administered systemically the therapeutic composition is preferably administered at a sufficient dosage to attain a blood level of antisense oligonucleotide from about 0.1 μM to about 10 μM . For localized administration, much lower concentrations than this may be effective, and much higher concentrations may be tolerated. One of skill in the art will appreciate that such therapeutic effect resulting in a lower effective concentration of the histone deacetylase inhibitor may vary considerably depending on the tissue, organ, or the particular animal or patient to be treated according to the invention.

In a preferred embodiment, the therapeutic composition of the invention is administered systemically at a sufficient dosage to attain a blood level of antisense oligonucleotide from about 0.01 μM to about 20 μM . In a particularly preferred embodiment, the therapeutic composition

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is administered at a sufficient dosage to attain a blood level of antisense oligonucleotide from about 0.05 μM to about 15 μM . In a more preferred embodiment, the blood level of antisense oligonucleotide is from about 0.1 μM to about 10 μM .

- 5 For localized administration, much lower concentrations than this may be therapeutically effective. Preferably, a total dosage of antisense oligonucleotide will range from about 0.1 mg to about 200 mg oligonucleotide per kg body weight per day. In a more preferred embodiment, a total dosage of antisense oligonucleotide will range from
10 about 1 mg to about 20 mg oligonucleotide per kg body weight per day. In a most preferred embodiment, a total dosage of antisense oligonucleotide will range from about 1 mg to about 10 mg oligonucleotide per kg body weight per day. In a particularly preferred embodiment, the therapeutically effective amount of a histone deacetylase antisense
15 oligonucleotide is about 5 mg oligonucleotide per kg body weight per day.

- In certain preferred embodiments of the third aspect of the invention, the method further comprises administering to the animal a therapeutically effective amount of a histone deacetylase small molecule inhibitor with a pharmaceutically acceptable carrier for a therapeutically
20 effective period of time. In some preferred embodiments, the histone deacetylase small molecule inhibitor is operably associated with the antisense oligonucleotide, as described *supra*.

- The histone deacetylase small molecule inhibitor-containing therapeutic composition of the invention is administered systemically at a
25 sufficient dosage to attain a blood level histone deacetylase small molecule inhibitor from about 0.01 μM to about 10 μM . In a particularly preferred embodiment, the therapeutic composition is administered at a sufficient dosage to attain a blood level of histone deacetylase small molecule

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inhibitor from about $0.05\mu\text{M}$ to about $10\mu\text{M}$. In a more preferred embodiment, the blood level of histone deacetylase small molecule inhibitor is from about $0.1\mu\text{M}$ to about $5\mu\text{M}$. For localized administration, much lower concentrations than this may be effective. Preferably, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.01 mg to about 100 mg protein effector per kg body weight per day. In a more preferred embodiment, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 50 mg protein effector per kg body weight per day. In a most preferred embodiment, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 10 mg protein effector per kg body weight per day. In a particularly preferred embodiment, the therapeutically effective synergistic amount of histone deacetylase small molecule inhibitor (when administered with an antisense oligonucleotide) is about 5 mg per kg body weight per day.

Certain preferred embodiments of this aspect of the invention result in an improved inhibitory effect, thereby reducing the therapeutically effective concentrations of either or both of the nucleic acid level inhibitor (*i.e.*, antisense oligonucleotide) and the protein level inhibitor (*i.e.*, histone deacetylase small molecule inhibitor) required to obtain a given inhibitory effect as compared to those necessary when either is used individually.

Furthermore, one of skill will appreciate that the therapeutically effective synergistic amount of either the antisense oligonucleotide or the histone deacetylase inhibitor may be lowered or increased by fine tuning and altering the amount of the other component. The invention therefore provides a method to tailor the administration/treatment to the particular exigencies specific to a given animal species or particular patient. Therapeutically effective ranges may be easily determined for example

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empirically by starting at relatively low amounts and by step-wise increments with concurrent evaluation of inhibition.

In a fourth aspect, the invention provides a method for identifying a specific histone deacetylase isoform that is required for induction of cell proliferation comprising contacting a cell with an agent of the first aspect of the invention. In certain preferred embodiments, the agent is an antisense oligonucleotide that inhibits the expression of a histone deacetylase isoform, wherein the antisense oligonucleotide is specific for a particular HDAC isoform, and thus inhibition of cell proliferation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is required for induction of cell proliferation. In other certain embodiments, the agent is a small molecule inhibitor that inhibits the activity of a histone deacetylase isoform, wherein the small molecule inhibitor is specific for a particular HDAC isoform, and thus inhibition of cell proliferation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is required for induction of cell proliferation. In certain preferred embodiments, the cell is a neoplastic cell, and the induction of cell proliferation is tumorigenesis. In still yet other preferred embodiments of the fourth aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In an fifth aspect, the invention provides a method for identifying a histone deacetylase isoform that is involved in induction of cell

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differentiation comprising contacting a cell with an agent that inhibits the expression of a histone deacetylase isoform, wherein induction of differentiation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is involved in induction of cell differentiation. In certain preferred embodiments, the agent is an antisense oligonucleotide of the first aspect of the invention. In other certain preferred embodiments, the agent is a small molecule inhibitor of the first aspect of the invention. In still other certain embodiments, the cell is a neoplastic cell. In still yet other preferred embodiments of the fifth aspect of the invention, the method comprises an agent of the first aspect of the invention which is a combination of one or more antisense oligonucleotides and/or one or more small molecule inhibitors of the first aspect of the invention. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In other certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

In a sixth aspect, the invention provides a method for inhibiting neoplastic cell growth in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of an agent of the first aspect of the invention. In certain embodiments thereof, the agent is an antisense oligonucleotide, which is combined with a pharmaceutically acceptable carrier and administered for a therapeutically effective period of time.

In certain embodiments where the agent of the first aspect of the invention is a histone deacetylase small molecule inhibitor, therapeutic compositions of the invention comprising said small molecule inhibitor(s) are administered systemically at a sufficient dosage to attain a blood level histone deacetylase small molecule inhibitor from about 0.01 μ M to about

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10 μM . In a particularly preferred embodiment, the therapeutic composition is administered at a sufficient dosage to attain a blood level of histone deacetylase small molecule inhibitor from about 0.05 μM to about 10 μM . In a more preferred embodiment, the blood level of histone

5 deacetylase small molecule inhibitor is from about 0.1 μM to about 5 μM . For localized administration, much lower concentrations than this may be effective. Preferably, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.01 mg to about 100 mg protein effector per kg body weight per day. In a more preferred embodiment, a total

10 dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 50 mg protein effector per kg body weight per day. In a most preferred embodiment, a total dosage of histone deacetylase small molecule inhibitor will range from about 0.1 mg to about 10 mg protein effector per kg body weight per day.

15 In a sixth aspect, the invention provides a method for investigating the role of a particular histone deacetylase isoform in cellular proliferation, including the proliferation of neoplastic cells. In this method, the cell type of interest is contacted with an amount of an antisense oligonucleotide that inhibits the expression of one or more specific histone deacetylase isoform,

20 as described for the first aspect according to the invention, resulting in inhibition of expression of the histone deacetylase isoform(s) in the cell. If the contacted cell with inhibited expression of the histone deacetylase isoform(s) also shows an inhibition in cell proliferation, then the histone deacetylase isoform(s) is required for the induction of cell proliferation. In

25 this scenario, if the contacted cell is a neoplastic cell, and the contacted neoplastic cell shows an inhibition of cell proliferation, then the histone deacetylase isoform whose expression was inhibited is a histone deacetylase isoform that is required for tumorigenesis. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2,

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HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7, or HDAC-8. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

Thus, by identifying a particular histone deacetylase isoform that is
5 required for in the induction of cell proliferation, only that particular histone deacetylase isoform need be targeted with an antisense oligonucleotide to inhibit cell proliferation or induce differentiation. Consequently, a lower therapeutically effective dose of antisense oligonucleotide may be able to effectively inhibit cell proliferation.
10 Moreover, undesirable side effects of inhibiting all histone deacetylase isoforms may be avoided by specifically inhibiting the one (or more) histone deacetylase isoform(s) required for inducing cell proliferation.

As previously indicated, the agent of the first aspect includes, but is not limited to, oligonucleotides and small molecule inhibitors that inhibit
15 the activity of one or more, but less than all, HDAC isoforms. The measurement of the enzymatic activity of a histone deacetylase isoform can be achieved using known methodologies. For example, Yoshida et al. (*J. Biol. Chem.* 265: 17174-17179, 1990) describe the assessment of histone deacetylase enzymatic activity by the detection of acetylated histones in
20 trichostatin A treated cells. Taunton et al. (*Science* 272: 408-411, 1996) similarly describes methods to measure histone deacetylase enzymatic activity using endogenous and recombinant HDAC. Both Yoshida et al. (*J. Biol. Chem.* 265: 17174-17179, 1990) and Taunton et al. (*Science* 272: 408-411, 1996) are hereby incorporated by reference.

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Preferably, the histone deacetylase small molecule inhibitor(s) of the invention that inhibits a histone deacetylase isoform that is required for induction of cell proliferation is a histone deacetylase small molecule inhibitor that interacts with and reduces the enzymatic activity of fewer
5 than all histone deacetylase isoforms.

In an seventh aspect, the invention provides a method for identifying a histone deacetylase isoform that is involved in induction of cell differentiation, comprising contacting a cell with an antisense oligonucleotide that inhibits the expression of a histone deacetylase
10 isoform, wherein induction of differentiation in the contacted cell identifies the histone deacetylase isoform as a histone deacetylase isoform that is involved in induction of cell differentiation. Preferably, the cell is a neoplastic cell. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6,
15 HDAC-7, or HDAC-8.

The phrase "inducing cell differentiation" and similar terms are used to denote the ability of a histone deacetylase antisense oligonucleotide or histone deacetylase small molecule inhibitor (or combination thereof) to induce differentiation in a contacted cell as compared to a cell that is not
20 contacted. Thus, a neoplastic cell, when contacted with a histone deacetylase antisense oligonucleotide or histone deacetylase small molecule inhibitor (or both) of the invention, may be induced to differentiate, resulting in the production of a daughter cell that is phylogenetically more advanced than the contacted cell.

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In an eighth aspect, the invention provides a method for inhibiting cell proliferation in a cell, comprising contacting a cell with at least two of the reagents selected from the group consisting of an antisense oligonucleotide that inhibits a specific histone deacetylase isoform, a
5 histone deacetylase small molecule inhibitor, an antisense oligonucleotide that inhibits a DNA methyltransferase, and a DNA methyltransferase small molecule inhibitor. In one embodiment, the inhibition of cell growth of the contacted cell is greater than the inhibition of cell growth of a cell contacted with only one of the reagents. In certain preferred embodiments, each of
10 the reagents selected from the group is substantially pure. In preferred embodiments, the cell is a neoplastic cell. In yet additional preferred embodiments, the reagents selected from the group are operably associated.

Antisense oligonucleotides that inhibit DNA methyltransferase are
15 described in Szyf and von Hofe, U.S. Patent No. 5,578,716, the entire contents of which are incorporated by reference. DNA methyltransferase small molecule inhibitors include, without limitation, 5-aza-2'-deoxycytidine (5-aza-dC), 5-fluoro-2'-deoxycytidine, 5-aza-cytidine (5-aza-C), or 5,6-dihydro-5-aza-cytidine.

20 In a ninth aspect, the invention provides a method for modulating cell proliferation or differentiation comprising contacting a cell with an agent of the first aspect of the invention, wherein one or more, but less than all, HDAC isoforms are inhibited, which results in a modulation of proliferation or differentiation. In preferred embodiments, the cell
25 proliferation is neoplasia.

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For purposes of this aspect, it is unimportant how the specific HDAC isoform is inhibited. The present invention has provided the discovery that specific individual HDACs are involved in cell proliferation or differentiation, whereas others are not. As demonstrated in this
5 specification, this is true regardless of how the particular HDAC isoform(s) is/are inhibited.

By the term "modulating" proliferation or differentiation is meant altering by increasing or decreasing the relative amount of proliferation or differentiation when compared to a control cell not contacted with an agent
10 of the first aspect of the invention. Preferably, there is an increase or decrease of about 10% to 100%. More preferably, there is an increase or decrease of about 25% to 100%. Most preferably, there is an increase or decrease of about 50% to 100%. The term "about" is used herein to indicate a variance of as much as 20% over or below the stated numerical values.

15 In certain preferred embodiments, the histone deacetylase isoform is selected from HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7 and HDAC-8. In certain preferred embodiments, the histone deacetylase isoform is HDAC-1.

The following examples are intended to further illustrate certain
20 preferred embodiments of the invention and are not limiting in nature. Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the
25 appended claims.

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EXAMPLES

Example 1

Synthesis and Identification of Antisense Oligonucleotides

5 Antisense (AS) and mismatch (MM) oligodeoxynucleotides (oligos) were designed to be directed against the 5'- or 3'-untranslated region (UTR) of the targeted gene. Oligos were synthesized with the phosphorothioate backbone and the 4X4 nucleotides 2'-O-methyl modification on an automated synthesizer and purified by preparative reverse-phase HPLC.

10 All oligos used were 20 base pairs in length.

 To identify antisense oligodeoxynucleotide (ODN) capable of inhibiting HDAC-1 expression in human cancer cells, eleven phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-1 gene (GenBank Accession No. U50079) were
15 initially screened in T24 cells at 100 nM. Cells were harvested after 24 hours of treatment, and HDAC-1 RNA expression was analyzed by Northern blot analysis. This screen identified HDAC-1 AS1 and AS2 as ODNs with antisense activity to human HDAC-1. HDAC-1 MM oligo was created as a control; compared to the antisense oligo, it has a 6-base
20 mismatch.

 Twenty-four phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-2 gene (GenBank Accession No. U31814) were screened as above. HDAC-2 AS was identified as an ODN with antisense activity to human HDAC-2. HDAC-2
25 MM was created as a control; compared to the antisense oligo, it contains a 7-base mismatch.

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Twenty-one phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-3 gene (GenBank Accession No. AF039703) were screened as above. HDAC-3 AS was identified as an ODN with antisense activity to human HDAC-3. HDAC-3
5 MM oligonucleotide was created as a control; compared to the antisense oligonucleotide, it contains a 6-base mismatch.

Seventeen phosphorothioate ODNs containing sequences complementary to the 5' or 3' UTR of the human HDAC-4 gene (GenBank Accession No. AB006626) were screened as above. HDAC-4 AS1 and AS2
10 were identified as ODNs with antisense activity to human HDAC-4. HDAC-4 MM1 and MM2 oligonucleotides were created as controls; compared to the antisense oligonucleotides, they each contain a 6-base mismatch.

Thirteen phosphorothioate ODNs containing sequences complementary to the 5' or 3' untranslated regions of the human HDAC-5
15 gene (GenBank Accession No. AF039691) were screened as above. HDAC-5 AS was identified as an ODN with antisense activity to human HDAC-5.

Thirteen phosphorothioate ODNs containing sequences complementary to the 5' or 3' untranslated regions of the human HDAC-6
20 gene (GenBank Accession No. AJ011972) were screened as above. HDAC-6 AS was identified as an ODN with antisense activity to human HDAC-6. HDAC-6 MM oligo was created as a control; compared to the antisense oligo, it contains a 7-base mismatch.

Eighteen phosphorothioate ODNs containing sequences complementary to the 5' or 3' untranslated regions of the human HDAC-7
25 gene (GenBank Accession No. AF239243) were screened as above. HDAC-7 AS was identified as an ODN with antisense activity to human HDAC-7.

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Fourteen phosphorothioate ODNs containing sequences complementary to the 5' or 3' untranslated regions of the human HDAC-8 gene (GenBank Accession No. AF230097) were screened as above. HDAC-8 AS was identified as an ODN with antisense activity to human HDAC-8.

5

Example 2 HDAC AS ODNs Specifically Inhibit Expression at the mRNA Level

In order to determine whether AS ODN treatment reduced HDAC expression at the mRNA level, human A549 cells were treated with 50 nM of antisense (AS) oligonucleotide directed against human HDAC-3 or its corresponding mismatch (MM) oligo for 48 hours, and A549 cells were treated with 50 nM or 100 nM of AS oligonucleotide directed against human HDAC-1, HDAC-2, HDAC-4, HDAC-5, HDAC-6 or HDAC-7 or the appropriate MM oligonucleotide (100 nM) for 24 hours.

Briefly, human A549 and/or T24 human bladder carcinoma cells were seeded in 10 cm tissue culture dishes one day prior to oligonucleotide treatment. The cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and were grown under the recommended culture conditions. Before the addition of the oligonucleotides, cells were washed with PBS (phosphate buffered saline). Next, lipofectin transfection reagent (GIBCO BRL Mississauga, Ontario, CA), at a concentration of 6.25 µg/ml, was added to serum free OPTIMEM medium (GIBCO BRL, Rockville, MD), which was then added to the cells. The oligonucleotides to be screened were then added directly to the cells (*i.e.*, one oligonucleotide per plate of cells). Mismatched oligonucleotides were used as controls. The same concentration of oligonucleotide (*e.g.*, 50 nM) was used per plate of cells for each oligonucleotide tested.

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Cells were harvested, and total RNAs were analyzed by Northern blot analysis. Briefly, total RNA was extracted using RNeasy miniprep columns (QIAGEN). Ten to twenty μ g of total RNA was run on a formaldehyde-containing 1% agarose gel with 0.5 M sodium phosphate (pH 7.0) as the buffer system. RNAs were then transferred to nitrocellulose membranes and hybridized with the indicated radiolabeled DNA probes. Autoradiography was performed using conventional procedures.

Figures 9A-9I present results of experiments conducted with HDAC-1 (Figure 9A), HDAC-2 (Figure 9B), HDAC-6 (Figure 9C), HDAC-3 (Figure 9D), HDAC-4 (Figures 9E and 9F), HDAC-5 (Figure 9G), HDAC-7 (Figure 9H), and HDAC-8 (Figure 9I) AS ODNs.

Treatment of cells with the respective HDAC AS ODN significantly inhibits the expression of the targeted HDAC mRNA in human A549 cells.

15

Example 3 HDAC OSDNs Inhibit HDAC Protein Expression

In order to determine whether treatment with HDAC OSDNs would inhibit HDAC protein expression, human A549 cancer cells were treated with 50 nM of paired antisense or its mismatch oligos directed against human HDAC-1, HDAC-2, HDAC-3, HDAC-4 or HDAC-6 for 48 hours. OSDN treatment conditions were as previously described.

Cells were lysed in buffer containing 1% Triton X- 100, 0.5 % sodium deoxycholate, 5 mM EDTA, 25 mM Tris-HCl, pH 7.5, plus protease inhibitors. Total protein was quantified by the protein assay reagent from Bio-Rad (Hercules, CA). 100 μ g of total protein was analyzed by SDS-PAGE. Next, total protein was transferred onto a PVDF membrane and probed with various HDAC-specific primary antibodies. Rabbit anti-

- 47 -

HDAC-1 (H-51), anti-HDAC-2 (H-54) antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) were used at 1:500 dilution. Rabbit anti-HDAC-3 antibody (Sigma, St. Louis, MO) was used at a dilution of 1:1000. Anti-HDAC-4 antibody was prepared as previously described (Wang, S.H. *et al.*, (1999) *Mol. Cell. Biol.* 19:7816-27), and was used at a dilution of 1:1000. Anti-HDAC-6 antibody was raised by immunizing rabbits with a GST fusion protein containing a fragment of HDAC-6 protein (amino acid #990 to #1216, GenBank Accession No. AAD29048). Rabbit antiserum was tested and found only to react specifically to the human HDAC-6 isoform. HDAC-6 antiserum was used at 1:500 dilution in Western blots to detect HDAC-6 in total cell lysates. Horse Radish Peroxidase conjugated secondary antibody was used at a dilution of 1:5000 to detect primary antibody binding. The secondary antibody binding was visualized by use of the Enhanced chemiluminescence (ECL) detection kit (Amersham-Pharmacia Biotech., Inc., Piscataway, NJ).

As shown in Figure 10A, the treatment of cells with HDAC-1, HDAC-2, HDAC-3, HDAC-4 or HDAC-6 ODNs for 48 hours specifically inhibits the expression of the respective HDAC isotype protein. Figure 10B presents dose dependent response for the inhibited expression of HDAC-1 protein in cells treated with two HDAC-1 AS ODNs. As predicted, treatment of cells with the respective mismatch (MM) control oligonucleotide does not result in a significant decrease in HDAC-1 protein expression in the treated cells.

In order to demonstrate that the level of HDAC protein expression is an important factor in the cancer cell phenotype, experiments were done to determine the level of HDAC isotype expression in normal and cancer cells. Western blot analysis was performed as described above.

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The results are presented in Table 3 clearly demonstrate that HDAC-1, HDAC-2, HDAC-3, HDAC-4, and HDAC-6, isotype proteins are overexpressed in cancer cell lines.

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Table 3
Expression Level of HDAC Isotypes in Human
Normal and Cancer Cells

<u>States of Cell</u>	<u>Tissue Type</u>	<u>Cell Designation</u>	<u>HDAC-1</u>	<u>HDAC-2</u>	<u>HDAC-3</u>	<u>HDAC-4</u>	<u>HDAC-6</u>
Normal	Breast Epithelial	HMEC	-	+	++	+	+
Normal	Foreskin Fibroblasts	MRHF	-	+	+	++	+
Cancer	Bladder	T24	+++	++	+++	++	+++
Cancer	Lung	A549	++	+++	+++	+++	++
Cancer	Colon	SW48	+++	+++	+++	+++	+++
Cancer	Colon	HCT116	++++	+++	+++	++++	+++
Cancer	Colon	HT29	+++	+++	+++	+++	+++
Cancer	Colon	NCI-H446	++	++++	+++	++++	++
Cancer	Cervix	Hela	+++	++++	+++	+++	+++
Cancer	Prostate	DU145	+++	+++	+++	++++	+++
Cancer	Breast	MDA-MB-231	++	+++	+++	+++	++++
Cancer	Breast	MCF-7	+++	+++	+++	++	++
Cancer	Breast	T47D	+++	+++	+++	++	+++
Cancer	Kidney	293T	+++	++++	++++	++	++
Cancer	Leukemia	K562	+++	++++	++++	++++	++++
Cander	Leukemia	Jurkat T	+++	++	++++	++	++

(-): not detectable; (+): detectable; (++) 2X over (+); (+++) 5X over (+); (++++): 10X over (+)

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Example 4

Effect of HDAC Isotype Specific OSDNs on Cell Growth and Apoptosis

5 In order to determine the effect of HDAC OSDNs on cell growth and cell death through apoptosis, A549 or T24 cells, MDAMB231 cells, and HMEC cells (ATCC, Manassas, VA) were treated with HDAC OSDNs as previously described.

 For the apoptosis study, cells were analyzed using the Cell Death
10 Detection ELISA^{Plus} kit (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's directions. Typically, 10,000 cells were plated in 96-well tissue culture dishes for 2 hours before harvest and lysis. Each sample was analyzed in duplicate. ELISA reading was done using a MR700 plate reader (DYNEX Technology, Ashford, Middlesex, England) at
15 410 nm. The reference was set at 490 nm.

 For the cell growth analysis, human cancer or normal cells were treated with 50 nM of paired AS or MM oligos directed against human HDAC-1, HDAC-2, HDAC-3, HDAC-4 or HDAC-6 for 72 hours. Cells were harvested and cell numbers counted by trypan blue exclusion using a
20 hemocytometer. Percentage of inhibition was calculated as $(100 - \text{AS cell numbers} / \text{control cell numbers})\%$.

 Results of the study are shown in Figures 11-13, and in Table 4 and Table 5. Treatment of human cancer cells by HDAC-4 AS, and to a lesser extent, HDAC 1 AS, induces growth arrest and apoptosis of various human
25 cancer. The corresponding mismatches have no effect. The effects of HDAC-4 AS or HDAC-1 AS on growth inhibition and apoptosis are significantly reduced in human normal cells. In contrast to the effects of HDAC-4 or HDAC-1 AS oligos, treatment with human HDAC-3 and HDAC-6 OSDNs has no effect on cancer cell growth or apoptosis, and

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treatment with human HDAC-2 OSDN has a minimal effect on cancer cell growth inhibition. Since T24 cells are p53 null and A549 cells have functional p53 protein, this induction of apoptosis is independent of p53 activity.

5

Table 4
Effect of HDAC Isotype-Specific OSDNs on Human Normal
and Cancer Cells Growth Inhibition (AS vs. MM)

	<u>Cancer</u> <u>Cells</u>	<u>Normal</u> <u>Cells</u>	MDAmb231	HMEC
	A549	T24		
HDAC-1 AS1	++(+)	+(+)	+/-	+/-
HDAC-2 AS	+(+)	+/-	-	+/-
HDAC-3 AS	-	-	-	-
HDAC-4 AS1	+++	++	++	+/-
HDAC-6 AS	-	-	+/-	-

"-": no inhibition, "+": <50% inhibition, "++": 50-75% inhibition,

"+++": >75% inhibition

10

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Table 5

Effect of HDAC Isotype-Specific OSDNs on Human Normal
and Cancer Cells Apoptosis After 48 Hour Treatment

5

	A549	T24	MDAmb231	HMEC
HDAC-1 AS1	+	-		-
HDAC-2 AS	-	-	-	-
HDAC-3 AS	-	-	-	-
HDAC-4 AS1	+++	+	++	-
HDAC-6 AS	-	-	-	-
TSA (100ng/ml)	++	++	++	+

"-": ≤ 2 x fold over non-specific background; "+": 2-3X fold; "++": 3-5X fold;

"+++": 5-8X fold; "++++": 8X fold

Example 5

10 Inhibition of HDAC Isoypes Induces the Expression of Growth Regulatory Genes

In order to understand the mechanism of growth arrest and
apoptosis of cancer cells induced by HDAC-1 or HDAC-4 AS treatment,

15 RNase protection assays were used to analyze the mRNA expression of cell
growth regulators (p21 and *GADD45*) and proapoptotic gene *Bax*.

Briefly, human cancer A549 or T24 cells were treated with HDAC
isotype-specific antisense oligonucleotides (each 50 nM) for 48 hours. Total
RNAs were extracted and RNase protection assays were performed to
20 analyzed the mRNA expression level of p21 and *GADD45*. As a control,
A549 cells were treated by lipofectin with or without TSA (250 ng/ml)
treatment for 16 hours. These RNase protection assays were done

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according to the following procedure. Total RNA from cells was prepared using "RNeasy miniprep kit" from QIAGEN following the manufacturer's manual. Labeled probes used in the protection assays were synthesized using "hStress-1 multiple-probe template sets" from Pharmingen (San Diego, California, U.S.A.) according to the manufacturer's instructions. Protection procedures were performed using "RPA IITM Ribonuclease Protection Assay Kit" from Ambion, (Austin, Tx) following the manufacturer's instructions. Quantitation of the bands from autoradiograms was done by using CycloneTM Phosphor System (Packard Instruments Co. Inc., Meriden, CT). The results are shown in Figures 14, 15 and Table 6.

Table 6

Up-Regulation of p21, *GADD45* and *Bax* After Cell
Treatment with Human HDAC Isotype-Specific Antisenses

	A549			T24		
	p21	<i>GADD45</i>	<i>Bax</i>	p21	<i>GADD45</i>	<i>Bax</i>
HDAC-1	1.7	5.0	0.8	2.4	3.4	0.9
HDAC-2	1.1	1.2	1.0	1.0	1.0	0.9
HDAC-3	0.7	0.9	1.0	0.9	1.0	1.0
HDAC-4	3.1	5.7	2.6	2.8	2.7	1.9
HDAC-6	1.0	1.0	1.0	1.0	0.8	1.1
TSA vs lipofectin	2.8	0.6	0.8			

Values indicate the fold induction of transcription as measured by RNase protection analysis for the respective AS vs. MM HDAC isotype-specific oligos.

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Results of the experiments are presented in Table 6. The inhibition of HDAC-4 in both A549 and T24 cancer cells dramatically up-regulates both p21 and *GADD45* expression. Inhibition of HDAC-1 by antisense oligonucleotides induces p21 expression but more greatly induces *GADD45* expression. Inhibition of HDAC-4, upregulates *Bax* expression in both A549 and T24 cells. The effect of HDAC-4 AS treatment (50 nM, 48 hrs) on p21 induction in A549 cells is comparable to that of TSA (0.3 to 0.8 uM, 16 hrs).

Experiments were also conducted to examine the affect of HDAC antisense oligonucleotides on HDAC protein expression. In A549 cells, treatment with HDAC-4 antisene oligonucleotides results in a dramatic increase in the level of p21 protein (Figure 15).

Example 6

Cyclin Gene Expression Is Repressed by HDAC-1 AS Treatment

Human cancer A549 cells were treated with AS1, AS2 or MM oligo directed human HDAC1 for 48 hours. Total cell lysates were harvested and analyzed by Western blot using antibodies against human HDAC1, cyclin B1, cyclin A and actin (all from Santa Cruz Biotechnology, Inc., Santa Cruz, California). AS1 or AS2 both repress expression of cyclin B1 and A. Downregulation of cyclin A and B1 expression by AS1 and AS2 correlates well with their ability to inhibit cancer cell growth. (Figure 16)

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Example 7

Inhibition of Growth in Soft Agar

5 1.3 g granulated agar (DIDFCO) was added to 100 ml deionized water and boiled in a microwave to sterilize. The boiled agar was held at 55°C until further use. Iscove's Modified Dulbecco's Medium (GIBCO/BRL), 100x Penicillin-Streptomycin-Glutamine (GIBCO/BRL) and fetal bovine serum (medicorp) were pre-warmed at 37°C. To 50 ml sterile
10 tubes was added 9 ml Iscove's medium, 2 ml fetal bovine serum and 0.2 ml 100x Pen-Strep-Gln. Then 9 ml 55°C 1.3% agar was added to each tube. The tube contents were mixed immediately, avoiding air bubbles, and 2.5 ml of the mixture was poured into each sterile 6 cm petri dish to form a polymerized bottom layer. Dishes with polymerized bottom layers were
15 then put in a CO₂ incubator at 37°C until further use. In 50 ml sterile tubes were prewarmed at 37°C for each 4 cell lines/samples, 20 ml Iscove's medium, 0.4 ml 100x Pen-Strep-Gln and 8 ml fetal bovine serum. Cells were trypsinized and counted by trypan blue staining and 20,000 cells were aliquotted into a sterile 15 ml tube. To the tube was then added DMEM
20 with low glucose (GIBCO/BRL) + 10% fetal bovine serum + Pen-Strep-Gln to a final volume of 1 ml. To the prewarmed 37°C mix in the 50 ml tube was quickly added 8 ml 55°C 1.3% agar, which was then mixed well. Nine ml of this mixture was then aliquotted to each 1 ml cells in the 15 ml tube which is then mixed and 5 ml aliquotted onto the polymerized bottom
25 layer of the 6 cm culture plates and allowed to polymerize at room temperature. After polymerization, 2.5 ml bottom layer mix was gently added over the cell layer. Plates were wrapped up in foil paper and

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incubated in a CO₂ incubator at 37°C for three weeks, at which time colonies in agar are counted. The results are shown in Figure 17.

These results demonstrate that an antisense oligonucleotide complementary to HDAC-1 inhibits growth of A549 cells in soft agar, but
5 antisense oligonucleotides complementary to HDAC-2 or HDAC-6, or mismatch controls, do not.

Example 8

Inhibition of HDAC Isotypes by Small Molecules

10

In order to demonstrate the identification of HDAC small molecule inhibitors, HDAC small molecule inhibitors were screened in histone deacetylase enzyme assays using various human histone deacetylase isotypic enzymes (*i.e.*, HDAC-1, HDAC-3, HDAC-4 and HDAC-6). Cloned
15 recombinant human HDAC-1, HDAC-3 and HDAC-6 enzymes, which were tagged with the Flag epitope (Grozing, C.M., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 96:4868-4873 (1999)) in their C-termini, were produced by a baculovirus expression system in insect cells.

Flag-tagged human HDAC-4 enzyme was produced in human
20 embryonic kidney 293 cells after transformation by the calcium phosphate precipitation method. Briefly, 293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and antibiotics. Plasmid DNA encoding Flag-tagged human HDAC-4 was precipitated by ethanol and resuspend in sterile water. DNA-calcium
25 precipitates, formed by mixing DNA, calcium chloride and 2XHEPES-buffered saline solution, were left on 293 cells for 12-16 hours. Cells were return to serum-contained DMEM medium and harvested at 48 hour post transfection for purification of Flag-tagged HDAC-4 enzyme.

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HDAC-1 and HDAC-6 were purified on a Q-Sepharose column, followed by an anti-Flag epitope affinity column. The other HDAC isotypes, HDAC-3 and HDAC-4, were purified directly on an anti-Flag affinity column.

5 For the deacetylase assay, 20,000 cpm of an [^3H]-metabolically-labeled acetylated histone was used as a substrate. Histones were incubated with cloned recombinant human HDAC enzymes at 37°C. For the HDAC-1 assay, the incubation time was 10 minutes, and for the HDAC-3, HDAC-4 and HDAC-6 assays, the incubation time was 2 hours. All assay conditions were pre-determined
10 to be certain that each reaction was linear. Reactions were stopped by adding acetic acid (0.04 M, final concentration) and HCl (250 mM, final concentration). The mixture was extracted with ethyl acetate, and the released [^3H]-acetic acid was quantified by liquid scintillation counting. For the inhibition studies, HDAC enzyme was preincubated with test compounds for 30 minutes at 4°C prior to the
15 start of the enzymatic assay. IC₅₀ values for HDAC enzyme inhibitors were identified with dose response curves for each individual compound and, thereby, obtaining a value for the concentration of inhibitor that produced fifty percent of the maximal inhibition.

20

Example 9

Inhibition of HDAC Activity in Whole Cells by Small Molecules

T24 human bladder cancer cells (ATCC, Manassas, VA) growing in culture were incubated with test compounds for 16 hours. Histones were
25 extracted from the cells by standard procedures (see *e.g.* Yoshida *et al.*, *supra*) after the culture period. Twenty µg total core histone protein was loaded onto SDS/PAGE and transferred to nitrocellulose membranes, which were then reacted with polyclonal antibody specific for acetylated histone H-4 (Upstate Biotech Inc., Lake Placid, NY). Horse Radish
30 Peroxidase conjugated secondary antibody was used at a dilution of 1:5000

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to detect primary antibody binding. The secondary antibody binding was visualized by use of the Enhanced chemiluminescence (ECL) detection kit (Amersham-Pharmacia Biotech., Inc., Piscataway, NJ). After exposure to film, acetylated H-4 signal was quantitated by densitometry.

- 5 The results, shown in Table 2 above, demonstrate that small molecule inhibitors selective for HDAC-1 and/or HDAC-4 can inhibit histone deacetylation in whole cells.

Example 10

10 Inhibition of Cancer Cell Growth by HDAC Small Molecule Inhibitors

- Two thousand (2,000) human colon cancer HCT116 cells (ATCC, Manassas, VA) were used in an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay to quantitatively determine cell proliferation and cytotoxicity. Typically, HCT116 cells were plated into each well of the 96-well tissue culture plate and left overnight to attach to the plate. Compounds at various concentrations were added into the culture media (final DMSO concentration 1%) and incubated for 72 hours. MTT solution (obtained from Sigma as powder) was added and incubated with the cells for 4 hours at 37°C in incubator with 5% CO₂. During the incubation, viable cells convert MTT to a water-insoluble formazan dye. Solubilizing buffer (50% N,N-dimethylformamide, 20% SDS, pH 4.7) was added to cells and incubated for overnight at 37C in incubator with 5% CO₂. Solubilized dye was quantitated by colorimetric reading at 570 nM using a reference of 630 nM. Optical density values were converted to cell number values by comparison to a standard growth curve for each cell line. The concentration test compound that reduces the total cell number to 50% that of the control treatment, i.e., 1% DMSO, is taken as the EC₅₀ value.
- 15
20
25

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The results, shown in Table 2 above, demonstrate that small molecule inhibitors selective for HDAC-1 and/or HDAC-4 can affect cell proliferation.

5

Example 11

Inhibition by Small Molecules of Tumor Growth in a Mouse Model

Female BALB/c nude mice were obtained from Charles River Laboratories (Charles River, NY) and used at age 8-10 weeks. Human prostate tumor cells (DU145, 2×10^6) or human colon cancer cells (HCT116; 2×10^6) or small lung core A549 2×10^6 were injected subcutaneously in the animal's flank and allowed to form solid tumors. Tumor fragments were serially passaged a minimum of three times, then approximately 30 mg tumor fragments were implanted subcutaneously through a small surgical incision under general anaesthesia. Small molecule inhibitor administration by intraperitoneal or oral administration was initiated when the tumors reached a volume of 100 mm^3 . For intraperitoneal administration, small molecule inhibitors of HDAC (40-50 mg/kg body weight/day) were dissolved in 100% DMSO and administered daily intraperitoneally by injection. For oral administration, small molecule inhibitors of HDAC (40-50 mg/kg body weight/days) were dissolved in a solution containing 65% polyethylene glycol 400 (PEG 400 (Sigma-Aldridge, Mississauga, Ontario, CA, Catalogue No. P-3265), 5% ethanol, and 30% water. Tumor volumes were monitored twice weekly up to 20 days. Each experimental group contained at least 6-8 animals. Percentage inhibition was calculated using volume of tumor from vehicle-treated mice as controls.

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The results, shown in Table 2 above, demonstrate that small molecule inhibitors selective for HDAC-1 and/or HDAC-4 can inhibit the growth of tumor cells *in vivo*.

5

Example 12

Upregulation of p21 Expression and Down regulation of Cyclin Gene Expression Following Treatment with Small Molecule Inhibitor

Sulfonamide aniline (compound 3, Table 2) is a small molecule
10 HDAC1 specific inhibitor. Human HCT116 cells were treated with escalating doses of compound 3 for 16 hours. Total cell lysates were harvested and expression of p21^{WAF1}, cyclin B1, cyclin A and actin was analyzed by Western blot. Ariti-p21^{WAF1} antibody was purchased from BD Transduction Laboratories (BD Pharmingen Canada, Missasagua, Ontario).
15 Compound 3 clearly upregulates expression of p21^{WAF1} and represses the expression of cyclin A and B1. The expression profile of these cell cycle regulators correlates well with the ability of compound 3 to inhibit HCT116 proliferation in MTT assays (see Table 2),

20

Example 13

Cell Cycle Arrest Induced by HDAC Small Molecule Inhibitors

Human cancer HCT116 cells were plated at 2×10^5 per 10-cm dish and were left to attach to the dish overnight in the incubator. Cells were
25 treated with small molecule inhibitors at various concentrations (1 uM and 10 uM, typically, dissolved in DMSO) for 16 hours. Cells were harvested by trypsinization and washed once in 1X PBS (phosphate buffered saline). The cells were resuspended in about 200ul 1X PBS and were fixed by slowly adding 1 ml 70% ethanol at -20°C and were left at least overnight at

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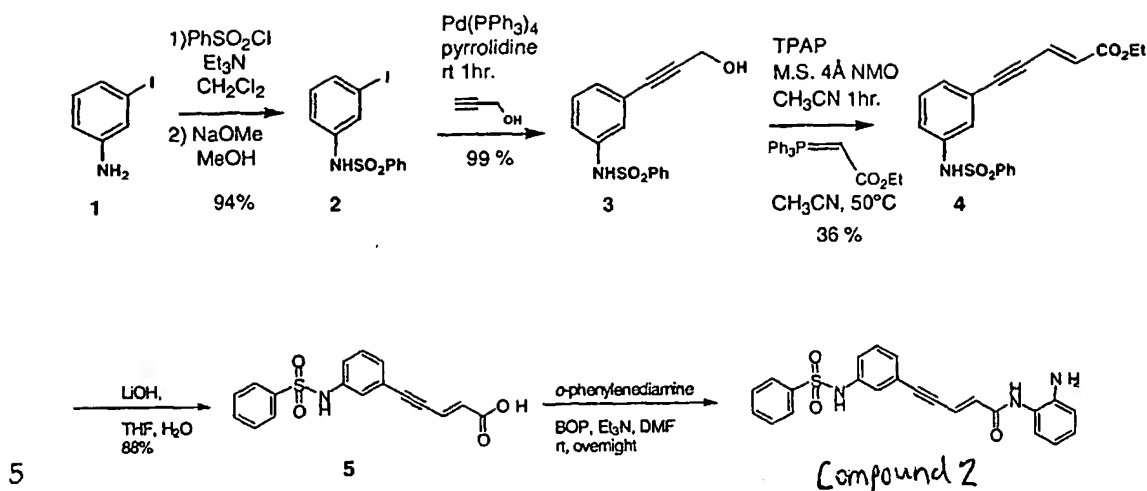
-20° C. Fixed cells were centrifuged at low speed (1,000 rpm) for 5 minutes, and the cell pellets were washed again with 1X PBS. Nucleic acids from fixed cells were incubated in a staining solution (0.1% (w/v) glucose in 1X PBS containing 50 ug/ml propidium iodide) (Sigma-Aldridge, 5 Mississauga, Ontario, CA) and RNase A (final 100 units/ml, (Sigma-Aldridge, Mississauga, Ontario, CA) for at least 30 minutes in the dark at 25° C. DNA content was measured by using a fluorescence-activated cell sorter (FACS) machine. Treatment of cells with all HDAC small molecule inhibitors in Table 2 results in a significant accumulation of cancer cell in 10 G2/M phase of the cell cycle and concomitantly reduce the accumulation of cancer cells in S phase of the cell cycle. The ratio of cells in G2/M phase vs. cells in the S phase was determined. The Effective concentration (EC) of a small molecule inhibitor to induce a (G2+M)/S ratio of 2.5 is calculated, as shown in Table 2.

15

Example: 14**Synthesis of Small Molecule Compound No. 2**

The following provides a synthesis scheme for small molecule Compound No. 2 from Table 2.

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Step 1: 3-(benzenesulfonylamino)-phenyl iodide (2)

To a solution of 3-iodoaniline (5 g, 22.8 mmol), in CH_2Cl_2 (100 mL), were added at room temperature Et_3N (6.97 mL) followed by benzenesulfonyl chloride (5.84 mL). The mixture was stirred 4 h then a white precipitate was formed. A saturated aqueous solution of NaHCO_3 was added and the phases were separated. The aqueous layer was extracted several times with CH_2Cl_2 and the combined extracts were dried over (MgSO_4) then evaporated. The crude mixture was dissolved in MeOH (100 mL) and NaOMe (6 g), was added and the mixture was heated 1 h at 60°C . The solution became clear with time and HCl (1N) was added. The solvent was evaporated under reduced pressure then the aqueous phase was extracted several times with CH_2Cl_2 . The combined organic extracts were dried over (MgSO_4) and evaporated. The crude material was purified by flash chromatography using (100% CH_2Cl_2) as solvent yielding the title compound **21** (7.68g, 94 %) as yellow solid.

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¹H NMR: (300 MHz, CDCl₃): δ 7.82-7.78 (m, 2H), 7.60-7.55 (m, 1H), 7.50-7.42 (m, 4H), 7.10-7.06 (m, 1H), 6.96 (t, J = 8Hz, 1H), 6.87 (broad s, 1H).

Step 2: 3-(benzenesulfonylamino)-phenyl-propargylic alcohol (3)

To a solution of 2 (500 mg, 1.39 mmol) in pyrrolidine (5 mL) at room
5 temperature was added Pd(PPh₃)₄ (80 mg, 0.069 mmol), followed by CuI (26
mg, 0.139 mmol). The mixture was stirred until complete dissolution.
Propargylic alcohol (162 •L, 2.78 mmol) was added and stirred 6 h at room
temperature. Then the solution was treated with a saturated aqueous
solution of NH₄Cl and extracted several times with AcOEt. The combined
10 organic extracts were dried over (MgSO₄) then evaporated. The residue
was purified by flash chromatography using hexane/AcOEt (1:1) as
solvent mixture yielding 3 (395 mg, 99 %) as yellow solid.

¹H NMR: (300 MHz, CDCl₃): δ 7.79-7.76 (m, 2H), 7.55-7.52 (m, 1H), 7.45 (t, J =
8Hz, 2H), 7.19-7.15 (m, 3H), 7.07-7.03 (m, 1H), 4.47 (s, 2H).

15

Step 3: 5-[3-(benzenesulfonylamino)-phenyl]-4-yn-2-pentenoate (4)

To a solution of 3 (2.75 g, 9.58 mmol) in CH₃CN (150 mL) at room
temperature were added 4-methylmorpholine N-oxide (NMO, 1.68 g, 14.37
mmol) followed by tetrapropylammonium perruthenate (TPAP, 336 mg,
20 .958 mmol). The mixture was stirred at room temperature 3 h, and then
filtrated through a Celite pad with a fritted glass funnel. To the filtrate
carbethoxymethylenetriphenyl-phosphorane (6.66 g, 19.16 mmol) was
added and the resulting solution was stirred 3 h at room temperature. The
solvent was evaporated and the residue was dissolved in CH₂Cl₂ and
25 washed with a saturated aqueous solution of NH₄Cl. The aqueous layer
was extracted several times with CH₂Cl₂, then the combined organic extract
were dried over (MgSO₄) and evaporated. The crude material was purified

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by flash chromatography using hexane/AcOEt (1:1) as solvent mixture giving 4 (1.21 g, 36%) as yellow oil.

¹H NMR: (300 MHz, CDCl₃): δ 7.81 (d, J = 8 Hz, 2H), 7.56-7.43 (m, 3H), 7.26-7.21 (m, 3H), 7.13-7.11 (m, 1H), 6.93 (d, J = 16 Hz, 1H), 6.29 (d, J = 16 Hz, 1H),
5 4.24 (q, J = 7 Hz, 2H), 1.31 (t, J = 7 Hz, 3H).

Step 4: 5-[3-(benzenesulfonylamino)-phenyl]-4-yn-2-pentenoic acid (5)

To a solution of 4 (888 mg, 2.50 mmol) in a solvent mixture of THF (10 mL) and water (10 mL) at room temperature was added LiOH (1.04 g, 25.01 mmol). The resulting mixture was heated 2 h at 60 °C and treated
10 with HCl (1N) until pH 2. The phases were separated and the aqueous layer was extracted several times with AcOEt. The combined organic extracts were dried over (MgSO₄) then evaporated. The crude residue was purified by flash chromatography using CH₂Cl₂/MeOH (9:1) as solvent mixture yielding 5 (712 mg, 88 %), as white solid.

15 ¹H NMR: (300 MHz, DMSO-*d*₆): δ 7.78-7.76 (m, 2H), 7.75-7.53 (m, 3H), 7.33-7.27 (m, 1H), 7.19-7.16 (m, 3H), 6.89 (d, J = 16 Hz, 1H), 6.33 (d, J = 16 Hz, 1H).

Step 5: Compound 2

Coupling of 5 with *o*-phenylenediamine in the presence of benzotriazol-1-yloxytris(dimethylamino)phosphonium
20 hexafluorophosphate (BOP) afforded the anilide **Compound 2**.

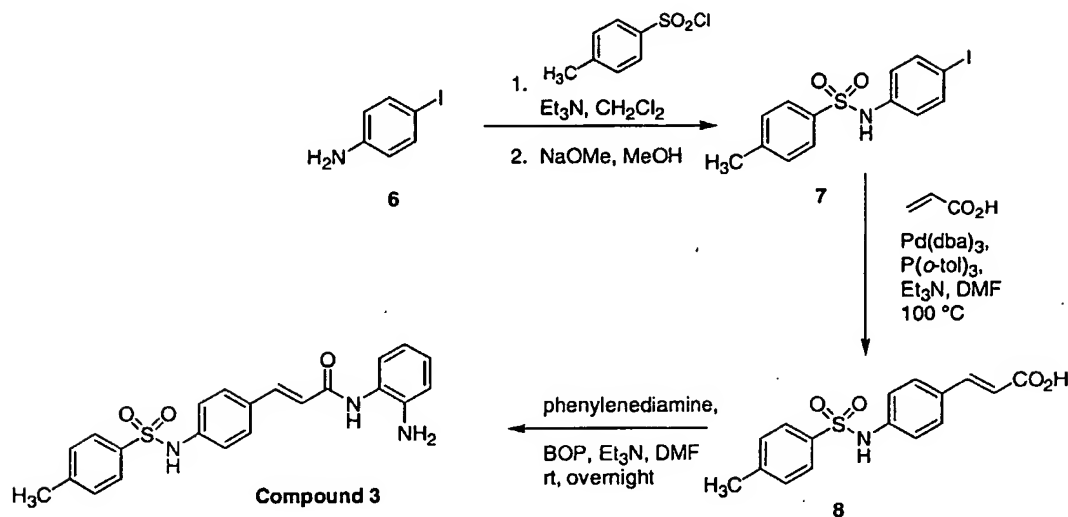
¹H NMR: (300 MHz, DMSO *d*₆): δ 7.77 (broad s, 4H); 7.57 (d, 1H, J=15.7 Hz); 7.35 (d, 1H, J=6.9 Hz); 7.03-6.94 (m, 6H); 6.76 (d, 1H, J=7.1 Hz); 6.59 (d, 1H, J=6.9 Hz); 4.98 (broad s, 2H); 2.19 (s, 3H).

25 ¹³C NMR: (75 MHz, DMSO *d*₆): δ 162.9; 141.6; 139.8; 139.0; 137.6; 134.8; 133.6; 129.6; 128.1; 127.3; 125.9; 125.4; 124.7; 123.2; 120.7; 116.2; 115.9; 20.3.

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Example : 15
Synthesis of Small Molecule Compound No. 3

- 5 The following provides a synthesis scheme for Compound No. 3 from Table 2.



10 Step 1: 3-[4-(toluenesulfonylamino)-phenyl]-2-propenoic acid (8)

- To a solution of 7 (1.39 mmol), in DMF (10 mL) at room temperature were added tris(dibenzylideneacetone)dipalladium(0) ($Pd_2(dba)_3$; 1.67 mmol), tri-*o*-tolylphosphine ($P(o\text{-tol})_3$, 0.83 mmol), Et_3N (3.48 mmol) and finally acrylic acid (1.67 mmol). The resulting solution was degassed and
- 15 purged several times with N_2 , then heated overnight at 100 °C. The solution was filtrated through a Celite pad with a fritted glass funnel then the filtrate was evaporated. The residue was purified by flash chromatography using CH_2Cl_2 /MeOH (95:5) as solvent mixture yielding the title compound 8.

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Step 2: N-Hydroxy-3-[4-(benzenesulfonylamino)-phenyl]-2-propenamide(Compound 3)

The acid 8 was coupled with *o*-phenylenediamine in the presence of benzotriazol-1-yloxytris(dimethylamino)phosphonium

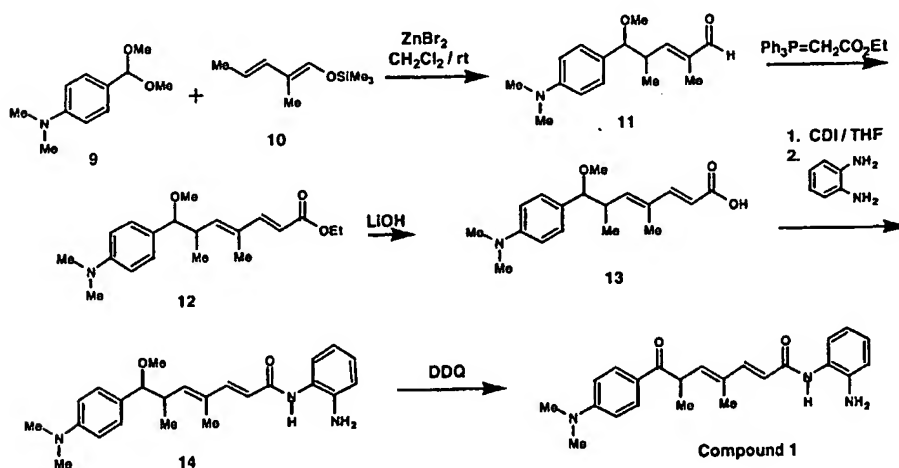
5 hexafluorophosphate (BOP) to afford the anilide **Compound 3**.

¹H NMR: (300 MHz, DMSO *d*₆): δ 7.77 (broad s, 4H); 7.57 (d, 1H, J=15.7Hz); 7.35 (d, 1H, J=6.9Hz); 7.03-6.94 (m, 6H); 6.76 (d, 1H, J=7.1 Hz); 6.59 (d, 1H, J=6.9Hz); 4.98 (broad s, 2H); 2.19 (s, 3H).

10 ¹³C NMR: (75 MHz, DMSO *d*₆): δ 162.9; 141.6; 139.8; 139.0; 137.6; 134.8; 133.6; 129.6; 128.1; 127.3; 125.9; 125.4; 124.7; 123.2; 120.7; 116.2; 115.9; 20.3.

Example : 16**Synthesis of Small Molecule No. Compound 1**

15 The following provides a synthesis scheme for small molecule Compound No. 1 from Table 2.



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Step 1: (11)

To a stirred solution of *p*-anisaldehyde dimethyl acetal (9) (10 mmol) in dry CH_2Cl_2 (60 mL) at rt was added 2-methyl-1-trimethylsilyloxypenta-1,3-diene (10) (*Tetrahedron*, 39: 881 (1983)) (10 mmol) followed by catalytic amount of anhydrous ZnBr_2 (25 mg). After being stirred for 5 h at rt, the reaction was quenched with water (20 mL). The two phases were separated and the aqueous layer was extracted with CH_2Cl_2 (2 \times 25 mL). The combined organic layers were washed with brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure.

Purification of the crude product by flash silica gel chromatography (25% ethyl acetate in hexane) afforded the desired aldehyde 11 in 68% yield as a mixture of two isomers in a ca. 2.5 : 1 ratio: **major isomer:** ^1H NMR (300 MHz, CDCl_3) δ 9.29 (s, 1H), 7.08 (d, J = 8.4 Hz, 2H), 6.67 (d, J = 8.4 Hz, 2H), 6.29 (dq, J = 9.9, 1.2 Hz, 1H), 3.96 (d, J = 6.6 Hz, 1H), 3.20 (s, 3H), 3.05 (m, 1H), 2.94 (s, 6H), 1.60 (d, J = 0.9 Hz, 3H), 1.12 (d, J = 6.9 Hz, 3H).

Step 2: (12)

A mixture of aldehyde 11 (5.14 mmol) and ethyl (triphenylphosphoranylidene)acetate (2.15 g, 6.16 mmol) in toluene (25 mL) was heated at reflux overnight under N_2 . After removal of the solvent under reduced pressure, the crude product obtained was purified by flash silica gel chromatography (10% ethyl acetate in hexane) to give the title compound 12 in 96 % yield as a mixture of two isomers in a ca. 2.5 : 1 ratio: **major isomer:** ^1H NMR (300 MHz, CDCl_3) δ 7.21 (dd, J = 15.6, 0.9 Hz, 1H), 7.06 (d, J = 8.7 Hz, 2H), 6.66 (d, J = 8.7 Hz, 2H), 5.69 (d, J = 15.6 Hz, 1H), 5.67 (br. d, J = 9.0 Hz, 1H), 4.17 (q, J = 7.2 Hz, 2H), 3.87 (d, J = 6.9 Hz, 1H), 3.18 (s, 3H), 2.93 (s, 6H), 2.81 (m, 1H), 1.59 (d, J = 1.2 Hz, 3H), 1.27 (t, J = 7.2 Hz, 3H), 1.05 (d, 6.6 Hz, 3H).

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Step 3: (13)

To a stirred solution of diene ester **12** (1.24 mmol) in methanol (10 mL) at rt was added aqueous LiOH 0.5 N solution (1.7mmol). After being stirred at 40 °C for 16 h, methanol was removed under reduced pressure and the resulting aqueous solution was acidified with 3N HCl (pH = ca. 4), extracted with ethyl acetate (25 × 3 mL), dried (MgSO₄), and concentrated under reduced pressure to give the desired carboxylic acid **13** in 98 % yield: **major isomer**: ¹H NMR (300 MHz, CD₃OD) δ 7.21 (d, J = 15.6, 0.6 Hz, 1H), 7.04 (d, J = 8.7 Hz, 2H), 6.70 (d, J = 8.7 Hz, 2H), 5.61 (d, J = 15.6 Hz, 1H), 5.60 (br. d, J = 10.0 Hz, 1H), 3.85 (d, J = 7.5 Hz, 1H), 3.13 (s, 3H), 2.87 (s, 6H), 2.81 (m, 1H), 1.52 (d, J = 1.5 Hz, 3H), 1.06 (d, J = 6.6 Hz, 3H).

Step 4: (14)

To a solution of carboxylic acid **13** (0.753 mmol) in anhydrous THF (10 mL) was added 1,1'-carbonyldiimidazole (0.790 mmol) at rt, and the mixture was stirred overnight. To the resulting solution was added 1,2-phenylenediamine (5.27 mmol), followed by trifluoroacetic acid (52 µl), and the reaction mixture was stirred for 16 h at rt. The reaction mixture was diluted with ethyl acetate (30 mL), washed with saturated NaHCO₃ solution (5 mL) and then water (10 mL), dried (MgSO₄), and concentrated. Purification by flash silica gel chromatography (50% ethyl acetate in toluene) afforded the title compound **14** in 61% yield, as a mixture of two isomers in a ca.3 : 1 ratio: **major isomer**: ¹H NMR (300 MHz, CD₃OD) δ 7.28-7.02 (m, 5H), 6.79 (m, 2H), 6.68 (d, J = 8.7 Hz, 2H), 5.83 (d, J = 15.0 Hz, 1H), 5.69 (d, J = 9.6 Hz, 1H), 3.87 (d, J = 6.9 Hz, 1H), 3.19 (s, 3H), 2.94 (s, 6H), 2.80 (m, 1H), 1.61 (br. s, 3H), 1.07 (d, J = 6.6 Hz, 3H).

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Step 5: (Compound 1)

To a stirred solution of compound 14 (0.216 mmol) in wet benzene (2 mL, benzene : H₂O = 9 : 1) at room temperature was added 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 0.432 mmol). After being stirred
5 vigorously for 15 min., the mixture was diluted with ethyl acetate (30 mL), washed with water (2 × 5 mL), dried (anhydr. MgSO₄), and concentrated. Purification by flash silica gel chromatography (50% ethyl acetate in hexanes, and then ethyl acetate only) afforded the title compound 35 (6 mg, 7% yield): ¹H NMR (300 MHz, CDCl₃) δ 7.83 (d, J = 9.0, 2H), 7.87 (br. s, 1H),
10 7.29 (d, J = 15.6 Hz, 1H), 7.27 (d, 7.8 Hz, 1H), 7.00 (m, 1H), 6.72 (m, 2H), 6.62 (d, J = 9.0 Hz, 2H), 5.97 (d, J = 15.6 Hz, 1H), 5.97 (d, J = 9.3 Hz, 1H), 4.34 (dq, J = 9.3, 6.9 Hz, 1H), 3.03 (s, 3H), 1.87 (br. s, 3H), 1.29 (d, J = 6.9 Hz, 3H); ¹³C NMR (75 MHz, CDCl₃)
δ 12.6, 17.6, 39.9, 40.8, 110.7, 118.0, 119.0, 119.3, 123.8, 124.4, 125.1, 126.9,
15 130.6, 132.5, 140.8, 146.2, 153.4, 164.8, 198.6.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific
20 embodimemts of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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What is claimed is:

1. An agent that inhibits one or more specific histone deacetylase isoforms, but less than all histone deacetylase isoforms.
- 5 2. The agent according to claim 1, wherein the agent that inhibits one or more specific histone deacetylase isoforms, but less than all histone deacetylase isoforms, is an oligonucleotide.
- 10 3. The oligonucleotide according to claim 2, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA that encodes a portion of one or more histone deacetylase isoforms.
- 15 4. The oligonucleotide according to claim 3, wherein the oligonucleotide is a chimeric oligonucleotide.
5. The oligonucleotide according to claim 3, wherein the oligonucleotide is a hybrid oligonucleotide.
- 20 6. The oligonucleotide according to claim 3, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA selected from the group consisting of
 - (a) a nucleic acid molecule encoding a portion of HDAC-1 (SEQ ID NO:2),
 - (b) a nucleic acid molecule encoding a portion of HDAC-2 (SEQ ID NO:4),
- 25

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- (c) a nucleic acid molecule encoding a portion of HDAC-3 (SEQ ID NO:6),
- (d) a nucleic acid molecule encoding a portion of HDAC-4 (SEQ ID NO:8),
- 5 (e) a nucleic acid molecule encoding a portion of HDAC-5 (SEQ ID NO:10),
- (f) a nucleic acid molecule encoding a portion of HDAC-6 (SEQ ID NO:12),
- (g) a nucleic acid molecule encoding a portion of HDAC-7 (SEQ ID NO:14), and
- 10 (h) a nucleic acid molecule encoding a portion of HDAC-8 (SEQ ID NO:18).

7. The oligonucleotide according to claim 6 having a nucleotide
15 sequence of from about 13 to about 35 nucleotides.

8. The oligonucleotide according to claim 6 having a nucleotide sequence of from about 15 to about 26 nucleotides.

20 9. The oligonucleotide according to claim 6 having one or more phosphorothioate internucleoside linkage, being 20-26 nucleotides in length, and being modified such that the terminal four nucleotides at the 5' end of the oligonucleotide and the terminal four nucleotides at the 3' end of the oligonucleotide each have 2'-O- methyl groups attached to their sugar
25 residues.

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10. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-1 (SEQ ID NO:2).
- 5 11. The oligonucleotide according to claim 10 that is SEQ ID NO:17 or SEQ ID NO:18.
12. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded
10 DNA encoding a portion of HDAC-2 (SEQ ID NO:4).
13. The oligonucleotide according to claim 12 that is SEQ ID NO:20.
- 15 14. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-3 (SEQ ID NO:6).
15. The oligonucleotide according to claim 14 that is SEQ ID
20 NO:22.
16. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-4 (SEQ ID NO:8).

25

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24. The oligonucleotide according to claim 6, wherein the oligonucleotide is complementary to a region of RNA or double-stranded DNA encoding a portion of HDAC-8 (SEQ ID NO:16).

5 25. The oligonucleotide according to claim 24 that is SEQ ID NO:32 or SEQ ID NO:33.

26. A method for inhibiting one or more histone deacetylase isoforms in a cell comprising contacting the cell with the agent according to
10 claim 1.

27. A method for inhibiting one or more histone deacetylase isoforms in a cell comprising contacting the cell with the oligonucleotide according to claim 3.
15

28. The method according to claim 27, wherein cell proliferation is inhibited in the contacted cell.

29. The method according to claim 27, wherein the
20 oligonucleotide that inhibits cell proliferation in a contacted cell induces the contacted cell to undergo growth retardation.

30. The method according to claim 27, wherein the oligonucleotide that inhibits cell proliferation in a contacted cell induces
25 the contacted cell to undergo growth arrest.

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31. The method according to claim 27, wherein the oligonucleotide that inhibits cell proliferation in a contacted cell induces the contacted cell to undergo programmed cell death.

5 32. The method according to claim 27, wherein the oligonucleotide that inhibits cell proliferation in a contacted cell induces the contacted cell to undergo necrotic cell death.

33. The method according to claim 27, further comprising
10 contacting the cell with a histone deacetylase small molecule inhibitor.

34. A method for inhibiting neoplastic cell proliferation in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of the
15 agent of claim 1.

35. A method for inhibiting neoplastic cell proliferation in an animal comprising administering to an animal having at least one neoplastic cell present in its body a therapeutically effective amount of the
20 oligonucleotide of claim 3.

36. The method according to claim 35, wherein the animal is a human.

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37. The method according to claim 35, further comprising administering to the animal a therapeutically effective amount of a histone deacetylase small molecule inhibitor with a pharmaceutically acceptable carrier for a therapeutically effective period of time.

5

38. A method for identifying a histone deacetylase isoform that is required for the induction of cell proliferation, the method comprising contacting the histone deacetylase isoform with an inhibitory agent, wherein a decrease in the induction of cell proliferation indicates that the
10 histone deacetylase isoform is required for the induction of cell proliferation.

39. The method according to claim 38, wherein the inhibitory agent is an oligonucleotide of claim 3.

15

40. A method for identifying a histone deacetylase isoform that is required for cell proliferation, the method comprising contacting the histone deacetylase isoform with an inhibitory agent, wherein a decrease in cell proliferation indicates that the histone deacetylase isoform is required
20 for cell proliferation.

41. The method according to claim 40, wherein the inhibitory agent is an oligonucleotide of claim 3.

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42. A method for identifying a histone deacetylase isoform that is required for the induction of cell differentiation, the method comprising contacting the histone deacetylase isoform with an inhibitory agent, wherein an induction of cell differentiation indicates that the histone
5 deacetylase isoform is required for the induction of cell proliferation.

43. The method according to claim 38, wherein the inhibitory agent is an oligonucleotide of claim 3.

10 44. A method for inhibiting cell proliferation in a cell, comprising contacting a cell with at least two reagents selected from the group consisting of an antisense oligonucleotide that inhibits a specific histone deacetylase isoform, a histone deacetylase small molecule inhibitor that inhibits a specific histone deacetylase isoform, an antisense oligonucleotide
15 that inhibits a DNA methyltransferase, and a DNA methyltransferase small molecule inhibitor.

45. A method for modulating cell proliferation or differentiation of a cell comprising inhibiting a specific HDAC isoform that is involved in
20 cell proliferation or differentiation by contacting the cell with an agent of claim 1.

46. The method according to claim 45, wherein the cell proliferation is neoplasia.

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47. The method according to claim 46, wherein the histone deacetylase isoform is selected from the group consisting of HDAC-1, HDAC-2, HDAC-3, HDAC-4, HDAC-5, HDAC-6, HDAC-7 and HDAC-8.

- 5 48. The method according to claim 47, wherein the histone deacetylase isoform is HDAC-1 and/or HDAC-4.

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MAQTQGTTRRKVCYYYDGDVGNYYYGQGHPMKPHRIRMTHNLLN
YGLYRKMEIYRPHKANAEEMTKYHSDDYIKFLRSIRPDNMSEYSKQMQRFNVEDCPV
FDGLFEFCQLSTGGSVASAVKLNKQQTDAVNWAGGLHHAKKSEASGFCYVNDIVLAI
LELLKYHQRVLYIDIDIHHGDGVEEAFYTTDRVMTVSFHKYGEYFPGTGDLRDIGAGK
GKYAVYPLRDGIDDESIEAIFKPVMSKVMEMFQPSAVVLQCGSDSLSGDRLGCFNL
TIKGHAKCVEFVKSFNLPMLMLGGGYTIRNVARCWTYETAVALDTEIPNELPYNDYF
EYFGPDFKLHISPSNMTNQNTNEYLEKIKQRLFENLRMLPHAPGVQMQAIPEDAIPEE
SGDEDEDDPKRISICSSDKRIACEEFSDEEEGEGGRKNSSNFKKAKRVKTEDEKE
KDPEEKKEVTETEEKPEAKGVKEEVKLA (SEQ ID NO:1)

FIG. 1A

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1 atgtctggg tctctgccc tctgtcact cgtgtgctgc tgttccccac tcggtcatcc tgagaacaca
 61 gcctgagcgr ctctgtcact cgggtagac cagcgggga cagcgggga ggagcaag atggcgaga
 121 cgcagggcac ccggaggaaa cggaggaaa gtctgttact actacgacgg gaatccgcat gactcataat ttgctgctca
 181 atggacaagg ccaccaatg ctaccgaaa cactacatta atgtcgttgc tcaacgttgg tgggcaagt gctgtgaaac
 241 actatggtct tgaccaaagta cagcagcag cagcaagcag atgcagagat cagttgtcta ctggtggttc tgggcaagt catgcaaga
 301 tgaccaaagta cagcagcag cagcaagcag atgcagagat cagttgtcta ctggtggttc tgggcaagt catgcaaga
 361 tgcggagta cagcagcag cagcaagcag atgcagagat cagttgtcta ctggtggttc tgggcaagt catgcaaga
 421 atggcctggt taaataagca gcagacggac atctggcttc ctgtacattg gacgggttca cgggggtg acattgatat tcaccatggt gacggcgtgg
 481 taaataagca gcagacggac atctggcttc ctgtacattg gacgggttca cgggggtg acattgatat tcaccatggt gacggcgtgg
 541 agtccgagg atctggcttc ctgtacattg gacgggttca cgggggtg acattgatat tcaccatggt gacggcgtgg
 601 taaagtatca cagaggggtg ctgtacattg gacgggttca cgggggtg acattgatat tcaccatggt gacggcgtgg
 661 aagaggcctt acttcccagg aactggggac gctccgagac agtaattggag atgttccagg ctagtgcggt ttagtgcggt tatggagagt
 721 acttcccagg aactggggac gctccgagac agtaattggag atgttccagg ctagtgcggt ttagtgcggt tatggagagt
 781 ttaactaccc gctccgagac agtaattggag atgttccagg ctagtgcggt ttagtgcggt tatggagagt
 841 tcatgtccaa agtaattggag atgttccagg ctagtgcggt ttagtgcggt tatggagagt
 901 actccctatc tggggatcgg tttacacattg ttaggttgc ttaggttgc ttaggttgc ttaggttgc ttaggttgc
 961 gtgtggaatt tttacacattg ttaggttgc ttaggttgc ttaggttgc ttaggttgc ttaggttgc ttaggttgc
 1021 ccatttcgtaa cgttgcccgg gcttccatag gcttccatag gcttccatag gcttccatag gcttccatag gcttccatag
 1081 tccctaataga gcttccatag gcttccatag gcttccatag gcttccatag gcttccatag gcttccatag gcttccatag
 1141 acatcagtcc tcccaatatg tcccaatatg tcccaatatg tcccaatatg tcccaatatg tcccaatatg tcccaatatg
 1201 agcagactgtt tgagaaacctt tgagaaacctt tgagaaacctt tgagaaacctt tgagaaacctt tgagaaacctt tgagaaacctt
 1261 ttcctgagga cgcctatccct cgcctatccct cgcctatccct cgcctatccct cgcctatccct cgcctatccct cgcctatccct
 1321 gcatctcgat ctgctcctct agaggggggc agaggggggc agaggggggc agaggggggc agaggggggc agaggggggc agaggggggc
 1381 aagaggaggg agaggggggc agaggggggc agaggggggc agaggggggc agaggggggc agaggggggc agaggggggc
 1441 aaacagagga tgaaaaagag tgaaaaagag tgaaaaagag tgaaaaagag tgaaaaagag tgaaaaagag tgaaaaagag
 1501 aaaccaaagga ggagaaagcca ggagaaagcca ggagaaagcca ggagaaagcca ggagaaagcca ggagaaagcca ggagaaagcca
 1561 tggacctctc cagctctggc ttcctgctga gtcctcagc tttcttccc c (SEQ ID NO:2)

FIG. 1B

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MAYSQGGKKCKVCYYDGDIGNYYYGQGHPMKPHRIRMTHNLLL
NYGLYRKMEIYRPHKATAEEMTKYHSDEYIKFLRSIRPDNMSEYSKQMHIPFNVGEDCP
AFDGLFEFCQLSTGGSVAGAVKLNRRQQTDMAVNWAGGLHHAKKYEASGFCYVNDIVLA
ILELLKYHQRVLYIDIDIHHRGDGVEEAFYTTDRVMTVSFYGEYFFPGTGLRDIGAG
KGKYAVNFPMPMGIDDESYGQIFKPIISKVMEYQPSAVVLQCGADSLSGDRLLGCFN
LTVKGHAKCVEVVKTFNLP LLMLGGGYTILRNVARCWTYETAVALDCEIPNELPYNDY
FEYFGPDFKLHISPSNMTNQNTPEYMEKIKQRLFENLRMLPHAPGVQMQAIPEDAVHE
DSGDEGEDPDKRISIRASDKRIACDEEFSDESEGEGRNVADHKKGAKARIEED
KKETEDKKTDVKEEDKSKDNSGEKTDTKGTKSEQLSNP (SEQ ID NO:3)

FIG. 2A

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1 cgcgagctt cggcacctc tggcggtgg tccggcgcc tccgagcct
61 ctccaccgg cctgccctc ccgcgggac tatcgcccc taccgagcct
121 ctctccggc cgagccgcg ccgagcagc agcagcagc tatcgcccc
181 gtggcgggg tggccggga ggcagcagc agcagcagc tatcgcccc
241 tgctactact acgacggtga tattggaaat tatttattatg taccgagcct
301 cctcatagaa tccgcatgac ccataacttg cgtttaaatt ctgttaaatt
361 gaaatatata ggcccatata agccactgcc gaagaaatga gaaagaaatga
421 tatatacaat ttctacggtc aataagacca gataacatgt gataacatgt
481 catataatta atgttgaga agattgtcca gcgtttgatg gctttaaatt
541 ctctcaactg gcggttcagt tgctggagct gtgaagttaa accgacaaca
601 gctgttaatt gggctggagg attacatcat gctaagaaat acgaagcatc
661 tacgttaatt atattgtgct tgccatcctt gaattactaa agtaccatca
721 tatatcgata tagatatcca tcatggtgat ggtgtcgaag aagcttttta
781 cgtgtaata gctgttcatt ccataaataat gggtgcgaag aagcttttta
841 agggatatatg gtgctgaaa aggcaaatat cctgctgcca ttcctggcac
901 atagacgatg agtcatatgg gcagatatat gcgtctgcca tatgctgcca
961 tatcaacctc atctaacagt caaagggtcat gctaaatgtg tagaagtgtt
1021 ggtgtgttca tactgatgct tggagggaggt ggtacacaa tccgtaatgt
1081 aacttaccat agactgcagt accagacttc aaactgcata ttagtccctc
1141 tggacatatg agtatttttg ctccagaata tacggaaaaa ataaacagc
1201 gattactttg atgacacttg tgtccagatg caagctatcc gagaagatgc
1261 aaccagaaca ctccagaata atgacacttg tgtccagatg gagaagatgc
1321 atgttacctc atgacacttg tgtccagatg gagaagatgc tttctattcg
1381 gacagtggag atgacacttg tgtccagatg gagaagatgc atgaaggaga
1441 aagcggatag ctgtgatga agaattctca gattctgagg atgaaggaga
1501 agaaatgttg ctgatcataa gaaaggagca agaaaggcta gaattgaaga
1561 gaaacagagg acaaaaaaac agacgttaag gaagaagata aatccaagga
1621 gaaaaaacag ataccaagg aaccaaatca gaacagctca gcaaccctg
1681 tctaccaat ttcagaaaat cattaaaaag aaaaatttga aaggaaaatg
1741 gaagacttct ggcttcattt tataactact tggcattgga tgtatttatt
1801 actttttcgt ttttgttttt ctgactactt ttatatgtga gattttctaa
1861 aaattttctt tctccaccat ctgggcaagt ttatatgtga gattttctaa
1921 gtcaaaaaaa ctgatctatt gctttatgtg atagtattta ttggtccttc
1981 aaag (SEQ ID NO:4) tgagctgaaa ttgagctgaaa

FIG. 2B

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MAKTVAIFYDPDVGNFHYGAGHPMKPHRLALTHSLVLHYGLYKK
MIVFKPYQASQHDMCRFHSEDIYDFLQRVSPTNMQFTKSLNAPNVGDDCPVFPGLFE
FCSRYTGASLQATQLNNKICDIANWAGGLHHAKKFEASGFCYVNDIVIGILELLKY
HPRVLYIDIDIHHGDGVQEAFFYLTDRVMTVSFHKYGNFFPGTGDMEVGAESGRYYC
LNVPLRDGIDDQSYKHLFQPVINQVDFYQPTCIVLQCGADSLGCDRLGCFNLSIRGH
CECVEYVKSFNIPPLLVLGGGYTVRNVARCWTYETSLLVEEAISEELPYSEYFEYFAP
DFTLHPDVSTRIENQSRQYLDQIRQTIFENLKMNLNAPSQVQIHDPADLLTYDRTDE
ADAEERGPEENYSRPEAPNEFYDGDHDNDKESDVEI (SEQ ID NO:5)

FIG. 3A

FIG. 3B

CD NO: 6)

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MLAMKHQQELLEHQRKLERHRQEQELEKQHQREKQLQQLKNKEKG
KESAVASTEVMKMLQEFVLNKKKALAHPNLNHCISSCPRTYWGKTQHSLLDQSSPPQS
GVSTSYNHPVLGMYDAKDDFPLRKTASEP NLKLSRLKQKVAERRSSPLRRKDGPPV
TALKKRPLDVTDSACSSAPGSGPSSPNNSSGVS AENGIAPAVPSIPAETSLAHLVA
REGSAAPLPYTSPLPNITLGLPATGPSAGTAGQQDTERLTLPALQQORLSLFPGTHL
TPYLSTSPLE RDGGAHSP LLQH MVLL EQPPAQA PLVTGLGALPLHAQSLVGADRVSP
SIHKLRQHRPLGRTQSAPLPQNAQALQHLV IQQQHQQFLEKHKQQFQQQQLQMNKIIP
KPSEPARQPESHPEEEEEELREHQALLDEPYLDRLPGQKEAHAQAGVQVKQEP IESDE
HEAEPPREVEPGORQPS EQLLEFRQALLLEQQRIHQLRNYQASMEAAGIPVSFGGHR
PLSRAQSSPASATFPVSVQEPPTKPRFTTGLVYDTMLKHKQCTCGSSSSHPEHAGRIQ
SIWSRLQETGLRGKCECIRGRKATLEELQTVHSEAH TLLYGTNPLNRQKLD SKKLLGS
LASVFVR LPPCGGVGVDSDTIWNEVHSAGAA RLAVGCVVVELVFKVATGELKNGFAVVRP
PGHHAEESTPMGFCYFNSVAVAAKLLQQORLSVSKILLVDWDVHHGNGTQQAFYSDPSV
LYMSLHRYDDGNFFPGSGAPDEVGTGPGVGFNVMNAFTGGLDPPMGDAEYLA AFRTVV
MPIASEFAPDVVLASSGFDAVEGHPTPLGGYNLSARCFGYLT KQLMGLAGGRIVLAL E
GGHDLTAICDASEACVSALLGNELDPLPEKVLQQRPNANAVRSM EKVM EIH SKYWRCL
QRTTSTAGRSLIEAQTCENE EAE TVTAMASLSVGVKPAEKRPDEEPMEEEPPL (SEQ ID NO: 7)

FIG. 4A

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```

1  ggaggtttgtg  ggcccgccgc  cgcggagcac  cgtcccccgc  gccgcccag  cccgagcccg
61  agcccgcgca  ccgcccgcg  ccgcccgcgc  cgcccgccgc  acagctccc  agcctgggcc
121  ccgggcgggc  ccgtggccgc  gtcccggctg  tcgcccgcgc  agcccagcc  cgcgcgccgc
181  cgggtggcgg  cgcaggctga  ggagatgcgg  cgcggagcgc  cggagcaggg  ctagagccgc
241  ccgcccgcgc  ccgcccgcgt  aagcgcagcc  ccggcccggc  gccgcgggc  catgtgccgc
301  cgcccgcgcc  gcgcccgcgc  cagcctgcag  gccttgagc  ccgcgccagg  tggacgccgc
361  cgggtccacac  ccgcccgcgc  cgcggccgtg  ggagcggggc  gccagcgtg  gccgcgcgcc
421  gtgggacccg  ccggtcccca  ggcccgccgc  gcccttctg  gaccttcca  ccgcgccgc
491  gaggcggctt  cgcccgcgcg  ggccggggcg  cgggggtggg  cacggcaggg  agcggcgccg
541  tctcccggtg  cggggcccgc  gccccccgag  caggttcac  tgcaagaagg  agcggacgcc
601  tctgttcaac  ttgtgggtta  cctggctcat  gagacctgc  cggcgaggct  cggcgcttga
661  acgtctgtga  ccagccctc  accgtcccgc  tacttgtatg  tgttggcggg  agtttgagc
721  tcgttgagc  tatcgtttcc  gtggaaattt  tgagccattt  cgaatcactt  aaaggagtgg
781  acattgctag  caatgagctc  ccaaagccat  ccagatggac  ttcttggccg  agaccagcca
841  gtggagctgc  tgaatcccgc  ccgcgtgaac  cacatgccc  gcacggtgga  tgtggccacg
901  gcgctgcctc  tgcaagtggc  ccccccgga  gegcccatgg  acccgcgct  ggaccaccag
961  ttctcactgc  ctgtggcaga  gccggccctg  cgggagcagc  agctgcagca  ggagctcctg

```

FIG. 4B-1

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1021 gcgctcaagc agaagcagca gatccagagg cagatcctca tcgccgagtt ccagagggcag
1081 cacgagcagc tctccggca gcacgaggcg cagctccacg agcacatcaa gcaataaacag
1141 gagatgctgg ccatgaagca ccagcaggag ctgctggaac accagcggaa gctggagagg
1201 caccgccagg agcaggagct ggagaagcag caccggagc agaagctgca gcagctcaag
1261 aacaaggaga agggcaaaaga gagtgccgtg gccagcacag aagtgaagat gaagttacaa
1321 gaatttgtcc tcaataaaaa gaaaggcgtg gccaccgga atctgaacca ctgcaattcc
1381 agagaccctc gctactggta cgggaaaaag cagcacagt cccttgacca gattcttcca
1441 cccagagcg gagtgtcgac ctctataac caccgggtcc tgggaatgta cgacggccaaa
1501 gatgacttcc ctcttaggaa aacagcttct gaaaccgaatc tgaatcacg gtccaggcta
1561 aagcagaaag tggccgaaag acggagcagc ccctgttac gcaggaaaga cgggccagtg
1621 gtcactgctc taaaaaagcg tccgttggat gtcacagact ccgcgtgcag cagcggccca
1681 ggctccggac ccagctcacc caacaaacagc tccgggagcg tcgcgtggag gaacgggtatc
1741 gcgcccgccg tcccagcat cccggcggag acgagtttgg cgcacagact tgtggcacga
1801 gaaggctcgg ccgctccact tccctctac acatgccat ccttgcccaa catcacgctg
1861 ggctgcctg ccacggccc ctctgcggc acggcgggccc agcaggacac cgagagactc
1921 accttcccc ccctccagca gaggctctcc ctbtccccg gcacccacct cactccctac
1981 ctgagcacct cgcccttggg gcgggacgga ggggcagcgc acagccctct tctgcagcac
2041 atggtcttac tggagcagc accggacaaa gcacccctcg tcacaggcct ggagtagtctg
2101 cccctccacg cacagtccct ggttggtgca gaccgggtgt cccctccat ccacaagctg
2161 cggcagcacc gccactggg gcggacccag tcggcccccg tgccccagaa cggccaggct
2221 ctgcagcacc tggatcatcca gcagcagcat cagcagtttc gcagagtttc tggagaaaca caagcagcag
2281 ttccagcagc agcaactgca gatgaacaag atcatcccca agccaagcga gccagcccgg
2341 cagccggaga gccacccgga ggagacggag gaggagctcc gtgagcacca ggctctgctg
2401 gacgagccct acctggaccg gctgccgggg cagaaggagg cgcacgcaca ggcggcgtg
2461 caggtgaagc aggagcccat tgagagcgtat gaggaagagg cagagcccc acgggaggtg
2521 gagccgggcc agcggccagc cagtgagcag gagctgctct tcagacagca agccctcctg
2581 ctggagcagc agcggatcca ccagctgagg aactaccagg cgtccatgga ggcggccggc
2641 atccccgtgt ccttcggcgg ccacaggcct ctgtccccgg cgcagtcctc accgcgtct
2701 gccaccttcc ccgtgtccgt gcaggagccc cccaccaagc cgaggttcac gacagggcctc
2761 gtgtatgaca cgctgatgct gaagcacccag tgcacctgcg ggagtagcag cagccacccc
2821 gaggacgccg ggaggatcca gagcatctgg tcccgccctg agaagacggg cctccggggc

FIG. 4B-2

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2881 aaatgcgagt gcatccgcgg acgcaaggcc accctggaag agctacagac ggtgcactcg
2941 gaagccacac cctcctgta tggcacgaac cccctcaacc ggcagaaact ggacagtaag
3001 aaacttctag gctcgctcgc ctccgtgttc gtccggctcc cttgcggtgg tgttgggggtg
3061 gacagtgaca ccataaggaa cgaggtgcac tcggcggggcagccgcct ggctgtgggc
3121 tgcgtggtag agctggtctt caaggtggcc acaggggagc tgaataatgg ctttgcctgtg
3181 gtccgcccc ctggacacca tgcggaggag agcacgccc a tgggcttttg ctacttcaac
3241 tccgcggccg tggcagccaa gcttctgcag cagaggttga gcgtgagcaa gatcctcatc
3301 gtggactggg acgtgcacca tggaaacggg acccagcagg ctttctacag cgaccctagc
3361 gtccctgtaca tgtccctcca ccgctacgac gatgggaaact tcttcccagg cagcggggct
3421 cctgatgagg tgggcacagg gcccggcgtg ggtttcaacg tcaacacggc tttcacccggc
3481 ggcctggacc ccccatggg agacgctgag tacttggcgg ccttcagaac ggttgtaatg
3541 ccgatacgcca gcgagtttgc ccggatgtg gtgctggtgt catcaggctt cgatgccgtg
3601 gagggccacc ccacccctct tgggggctac aacctctccg ccagatgctt cgggtacctg
3661 acgaagcagc tgatgggctt ggctggcggc cggattgtcc tggccctcga gggaggccac
3721 gacctgaccg ccatttgcga cgctcggaa gcatgtgttt ctgccttgct tggaaacgag
3781 cttgatcctc tccagaaaa ggttttacag caaagaccca atgcaaacgc tgtccgttcc
3841 atggagaaa tgatggagat ccacagcaag tactggcgtt gcctgcagcg cacaacctcc
3901 acagcggggc gttctctgat cgaggctcag acttgcgaga acgaagaagc cgagacggtc
3961 accgccatgg cctcgctgtc cgtggacgtg aagcccgccg aaaaagagacc agatgaggag
4021 cccatgggaag aggagccgc cctgtagcac tccctcgaa gtgctgttct cttgtctgtc
4081 tgtctctgtc ttgaagctca gccaaagaaac tttcccggtt cagcctgctg tcccaccgtg
4141 gggctctctt ggagcaccca gggacaccca gcgtgcaaca tgcacgggaa gccttcttgc
4201 cgcccaaggc cacaggcttc gagacgcaca tgcacgcctg ggcgtggcag cctcacaggg
4261 aacacgggac agacgccggc gacgcgcaga cacacggaca cgcggaaagc aagcacactc
4321 tggcgggtcc cgcaaggggc gccgtggaa gcaagagcct aaaggaacag gcggccgagc
4381 tgcggaattc agttgacacg aggcacagaa acaaaatatc aaagatctaa taatacaaaa
4441 caaacttgat taaaactggt gcttaaaagt tattaccac aactccacag tctctgtgta
4501 aaccactcga ctcatcttgt agcttatttt tttttttaaag aggacgtttt ctacggctgt
4561 ggcccgcttc tgtgaaccat agcgggtgtgc ggcgggggggt ctgcaccccg gtgggggaca
4621 gagggacctt taaagaaaaa aaaactggac agaaacagga atgtgagctg ggggagctgg
4681 cttgagtttc tcaaaagcca tcggaagatg cgagtttgtg cttttttttt tattgctctg

FIG. 4B-3

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4741 gtggattttt gtggctgggt tttctgaagt ctgaggaaca atgccttaag aaaaaacaaa
4801 cagcaggaat cggtaggaca gtttcctgtg gccagccgag cctggcagtg ctggcacccg
4861 gagctggcct gacgcctcaa gacggggcac gacccgtcat ctccggggcc aggggctgca
4921 gcccgcggtt cctgtttttg ctttatgtct gtttaagaaa aatggaggta gttccaaaaa
4981 agtggcaaat cccgttggag gttttgaagt ccaacaaatt taaacgaat ccaaagtgtt
5041 ctacacgtc acatcagatt gagcatctcc atctggctct gaagcatgtg tagggcacac
5101 ttgcagtgtt acgatcggaa tgctttttat taaaagcaag tagcatgaag tatgtcttaa
5161 attttaggta taaataaata tataatgta taatatatat tccaatgtat tccaagctaa
5221 gaaacttact tgattcttat gaaatcttga taaaatattt ataatgcatt tatagaaaaa
5281 gtatatatat atatatataa tgaatgcaga ttgcgaaggt ccctgcaaat ggatggcttg
5341 tgaatttgct ctcaaggtgc ttatggaaag ggatccctgat tgattgaaat tcatgttttc
5401 tcaagctcca gattggctag atttcagatc gccaacacat tcgccactgg gcaactaccc
5461 tacaagtttg tactttcatt ttaattattt tctaacagaa ccgctccctt ctccaagcct
5521 tcatgcacat atgtacctaa tgagttttta tagcaagaa tataaatgtg ctgttgattt
5561 ttgtatgaat tttttcacaa aaagatcctg aataagcatt gttttatgaa ttttacattt
5641 ttccctcacca tttagcaatt ttccgaatgg taataatgtc taaatctttt tctttctga
5701 attcttgctt gtacattttt ttttaccttt caaaggtttt taattatttt tgtttttatt
5761 tttgtacgat gatttttctg cagcgtacag aattgttgct gtcagattct attttcagaa
5821 agtgagagga gggaccgtag gtcttttctg agtgacacca acgatttgtt ctttcctggt
5881 ctgtccctagg agctgtataa agaagcccag gggctctttt taactttcaa cactagtagt
5941 attacgagg gtggtgtgtt ttccctctcc gtggcaaggg cagggagggt tgcttaggat
6001 gcccgccac cctgggaggc ttgccagatg ccggggggcag tcagcattaa tgaactcat
6061 gtttaaaactt ctctgaccac atcgtcagga tagaatctta acttgagttt tccaaacacc
6121 ttttagcat gtcagcaatg catggggcac acgtggggct ctttacccac ttgggttttt
6181 ccaactgcag cactggcca gccctggatt ttggagcctg tggctgcaag gaaccaggg
6241 acccttgttg cctggtgaac ctgcagggag ggtatgatg cctgaccagg acagccagtc
6301 ttactctttt ttctcttcaa cagtaactga cagtcacgtt ttactggtaa cttattttcc
6361 agcacatgaa gccaccagtt tcatctccaaa gtgtatatgt ggtcagact tgggggcaga
6421 agttcagaca caccgtgctc aggagggacc cagagccgag ttctcgagtt tggtaagt
6481 tacagggtag cttctgaaat taactcaaac ttttgaccaa atgagtgag atctctggat
6541 tcaacttggtc actgggctgc tgatgggtcag ctctgagaca gtggtttgag agcaggcaga

FIG. 4B-4

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FIG. 4B-5

6601 acggtcttgg gactgtttg actttcccc cctgggtggc cactcttgc tctgaagccc
6661 agattggcaa gaggagctgg tccattcccc attcatggca cagaacagtg gcagggccca
6721 gctagcaggc tttcttggcc tcttggccct cattctctgc atagccctct ggggacccctg
6781 ccacctgccc tcttaccctg tcttaccctg cgtggctta tggggaggaa tgcatactct cactttttt
6841 ttttaagcag atgatgggat aacatggact aacatggact gctcagtggc caggttatca gtggggggac
6901 ttaattctaa tctcattcaa atggagacga cctctgcaaa cctctggcag ggggaggcaa
6961 gtttcatttg ttagctcact ccagcttcac aaatgtgctg agagcattac tgtgtagcct
7021 tttctttgaa gacacactcg gctcttctcc acagcaagcg tccaggggcag atggcagagg
7081 atctgcctcg gcgtctgcag gcgggaccac gtcagggagg gtcccttcat gtgttctccc
7141 tgtgggtcct tggaccttta gcttttttct tcccttgcaa aggccttggg ggcactggct
7201 gggagtcagc aagcgagcac ttatatccc tttaggggaa accctgatga cgcactggg
SUBSTITUTE SHEET (RULE 26) 7261 cctcttggcg tctgacctgc cctgcctgct tcccgccgtg ccgacgctg ccacgtgcc
7321 caccgcccac cagcaggcgg ctgccccgga ggcgtggcc cgtgggact ggccgcccc
7381 cccagcgtc ccagggtctt ggtctggag ggccacttg tcaagggtt tcagttttc
7441 tttacttctt ttgaaaatct gtttgcaagg ggaaggacca ttctgtaatg gtctgacaca
7501 aaagcaagtt tgatttttgc agcactagca atggactttg ttgcttttct ttttgatcag
7561 aacattcctt ctttactggt cacagccag tgcctatcc attcttctt ttgtagactt
7621 tgggcccacg tgttttatgg gcattgatac atataaat atataatat aatatatat
7681 gaatacattt ttttaagttt cctacacctg gaggttgcac ggactgtacg accggcatga
7741 ctttatattg tatacagatt ttgcacgcca aactcggcag ctttgggaa gaagaaaaat
7801 gcctttctgt tcccctctca tgacatttgc agatacaaaa gatggaaat ttctgtaaa
7861 acaaaacctt gaaggagagg agggcgggga agtttgcgtc ttattgaaat tattcttaag
7921 aaattgtact ttttatgta agaaaaataa aaaggactac ttaaacattt gtcatattaa
7981 gaaaaaaagt ttatctagca cttgtgacat accaataata gagtttattg tatttatgtg
8041 gaaacagtgt tttagggaaa ctactcagaa ttcacagtga actgcctgtc tctctcagat
8101 tgatttggag gaattttgtt ttgttttgtt ttgtttgttt ccttttatct cctccacgg
8161 gccaggcgag cgcgcccgc cctcactggc cttgtgacgg ttattctga ttgagaactg
8221 ggcggactcg aaagagtccc cttttccgca cagctgtgtt gactttttaa ttacttttag
8281 gtgatgtatg gctaagattt cactttaagc agtcgtgaac tgtgcgagca ctgtggttta
8341 caattatact ttgcatacga aggaaacccat ttcttcatgg taacgaagct gagcgtgttc
8401 ttagctcggc ctcactttgt ctctggcatt gattaaaagt ctgctattga aagaaaaag (SEQ ID NO: 8)

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LRQGGTLTGKFMSTSSIPGCLLGVALEGDGSPHGHASLLQHVL
LEQARQQSTLIAVPLHGQSPLVTGERVATSMRTVGKLP
RHRPLSRTQSSPLPQSPQAL
QQLVMQQHQHFLEKQKQQLQLGKILTKTGELPRQPT
THPEETEELTEQQEVLLGE
GALTMPREGSESTQEDLEEEDEEDGEEEDCIQVKDEEGES
GAEEGPDLEEPGA
GYKKLFSDAQPLQPLQVYQAPLSLATVPHQALGRTQSS
PAAAPGGMKSPPDQPVKHLFT
TGVVYDTFMLKHQCMCGNTHVHPEHAGRIQSIWSRLQ
ETGLLSKCERIRGRKATLDEI
QTVHSEYIHTLLYGTSPLNQRKLD SKKLLGPI SQKMYAV
LPCGGIGVDSDTVWNEMHSS
SAVRMAVGCLLELAFKVAAGELKNGFAIIRPPGHAAEES
TAMGFCFFNSVAITAKLLQ
QKLNVGKVLIVDWDIHHGNGTQQAFYNDPSVLYISLHRYD
NGNFFPGSGAPEEVGGGP
GVGYNVNVAWTGGVDPPIGDVEYLTAFRTVVMPIAHEFSP
DVVTVLSAGFDAVEGHLSP
LGGYSVTARCFGHLTRQLMTLAGGRVVLALLEGGHDLT
AICDASEACVSALLSVELQPL
DELVLQOKPNINAVATLEKVIETQSKHWSCVQKFAAGLGR
SLREAQAGETEEAETVSA
MALLSVGAEQAAAAAREHSPRAEPEMEQEPAL (SEQ ID NO:9)

FIG. 5A

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1  ccctgcggca  gggtaggcacg  ctgaccgggca  agttcatgag  cacatcctct  attcctggct
61  gcctgctggg  cgtggcactg  gaggcgacg  ggagcccca  cgggcatgcc  tccctgctgc
121 agcatgtgct  gttgctggag  caggcccgcc  agcagagcac  cctcatgtct  gtgccactcc
181 acgggcagtc  cccactagtg  acgggtgaac  gtgtggccac  cagcatgcgg  acggtaggca
241 agctcccgcg  gcatcggccc  ctgagccgca  ctcagtcctc  accgctgccg  cagagtcccc
301 aggccctgca  gcagctggtc  atgcaacaac  agcaccagca  gttcctggag  aagcagaagc
361 agcagcagct  acagctgggc  aagatcctca  ccaagacagg  ggagctgcc  aggcagccca
421 ccaccacccc  taggagaca  gaggaggagc  tgacggagca  gcaggaggtc  ttgctggggg
481 agggagccct  gaccatgccc  cgggagggct  ccacagagag  tgagagcaca  caggaagacc
541 tggaggagga  ggacgaggaa  gaggatgggg  aggaggagga  ggattgcata  caggttaagg
601 acgaggaggg  cgagagtggc  gctgaggagg  ggcccgaact  ggaggagcct  ggtgctggat
661 acaaaaaact  gttctcagat  gccagccgc  tgacgccttt  gcagggtgtac  caggcgcccc
721 tcagccctggc  cactgtgccc  caccaggccc  tgggccgtac  ccagtcctcc  cctgctgccc
781 ctggggggcat  gaagagcccc  ccagaccagc  ccgtcaagca  cctcttcacc  acagggtgtg
841 tctacgacac  gttcatgcta  aagcaccagt  gcatgtgcgg  gaacacacac  gtgcaccctg
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FIG. 5B-1

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901 agcatgctgg ccggtaccag agcatctggt cccggctgca ggagacaggc ctgcttagca
961 agtgcgagcg gatccgaggt cgcaaaagcca cgctagatga gatccagaca gtgcactctg
1021 aataccacac cctgctctac ggacaccagtc cctcaaccg gcagaagcta gacagcaaga
1081 agttgctcgg ccccatcagc cagaagatgt atgctgtgct gccttgtggg ggcacgggg
1141 tggacagtga caccgtgtgg aatgagatgc actcctccag tgctgtgcgt atggcagtgg
1201 gctgcctgct ggagctggcc ttcaaggagg ctgcaggaga gctcaagaat ggatttgcca
1261 tcatccggcc ccaggacac caccggagg aatccacagc cacgggattc tgcttcttca
1321 actctgtagc catcacggca aaactcctac agcagaagtt agcgtgggc aaggtcctca
1381 tcgtggactg ggacattcac catggcaatg gacccagca ggcgttctat aatgacccct
1441 ctgtgctcta catctctctg catcgctatg acaacgggaa cttctttcca ggctctgggg
1501 ctcctgaaga ggtggtgga ggaccaggcg tggggtacaa tgtgaacgtg gcatggacag
1561 gaggtgtgga ccccccatt ggagacgtgg agtaccttac agccttcagg acagtgggtga
1621 tgcccatcgc ccacgagttc tcacctgatg tggctcctagt ctccgcccggg tttgatgctg
1681 ttgaaggaca tctgtctcct ctgggtggct actctgtcac cgccagatgt tttggccact
1741 tgaccaggca gctgatgacc ctggcagggg gccgggtggt gctggcccctg gagggaggcc
1801 atgacttgac cgccatctgt gatgcctctg aggcttgtgt ctccggctctg ctcagtgtag
1861 agctgcagcc ctggatgag gcagtcttc agcaaaagcc caacatcaac gcagtggcca
1921 cgctagagaa agtcatcgag atccagagca aacactggag ctgtgtgcag aagttcgccg
1981 ctggtctggg ccggtcccctg cgagaggccc aagcaggtga ggccgaggag gccgagactg
2041 tgagcgccat ggccttgctg tcggtggggg ccgagcaggc ccaggctgcg gcagccccggg
2101 aacacagccc caggccggca gaggagccca tggagcagga gcctgccctg tgacgccccg
2161 gcccccatcc ctctcggtt caccattgtg attttgttta ttttttctat taaaaacaaa
2221 aagtcacaca ttc (SEQ ID NO:10)

FIG. 5B-2

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1 mtstgqdstt trqrrsrqnp qspqgdssvt skrnikkav prsipnlaev kkkgkmmkklg
 61 gameedlivg lqgmdlnlea ealagtglvl deqlnefhcl wddsfpegpe rlhaikqgli
 121 qeglldrcvs fgarfaekee lmlvhsleyi dlmcttqymn egelrvladt ydsvylhpnns
 181 yscaclasgs vlrlvdavlg aeirngmai i rppghhaqhs lmdgycmfh vavaaryaqq
 241 khrrirrvliv dwdvhhgqgt qftfdqdpv lyfsihryeq grfwphlkas nwsttgfgqg
 301 qgytinvpwn qvgmrdadyi aafhlvllpv alefqpqlvl vaagfdalqg dpkgemaatp
 361 agfaqlthll mglaggklil slaggynira laegvsaslh tllgdpcpml espgapcrsa
 421 qasvscalea lepfwevlvr stetverdnm eednveesee egpweppvlp iltwvvlqsr
 481 tglvydqnmn nhcnlwdshh pevpriliri morleelgia grcltittprp ateaelltch
 541 saeyvghlra tekmtrelh ressnfdsiy icpstfacaq iatgaacrly eavisgevin
 601 gaavvrppgh haeqdaacgf cffnsavava rhaqtisgha lrilivdwdv hhngngtqhmf
 661 eddpsvlyvs lhrydhgtff pmgdegassq igraagtgt vnvaungprm gdadylaawh
 721 rlvlpiafef npelvlvsag fdaargdplg gcqvspegya hlthllmgl sgrilileg
 781 gynltsises maactrsilg dppplltlpr pplsgalasi tetiqvhrry wrslrvmkve
 841 dregpssskl vtkkapqpak prlaermtrr ekkvleagmg kvtsasfgee stpgqtnset
 901 avvalcqddp seaatggatl aqtiseaaig gamlgqttse eavggatpdpq ttseetvsga
 961 ildqttse da vggatigqtt seeavggatl aqtiseaame gatldqttse eapgggteliq
 1021 tplasstdhq tpptspvqgt tpqispstli gslrtlelgs esqgasesqa pgeenllgea
 1081 agggqdmadsm lmqgsrgltd qaiyfayvtp lwcphlvavc pipaagldvt qpcgdcgtiq
 1141 enwvclscyq vycgryingh mlqhhgnsgh plvlsyidls awcyycqayv hhqalldvkn
 1201 iahqnkfged mphph (SEQ ID:11)

FIG. 6A

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```
1  gggcagtccc  ctgaggagcg  gggctggttg  aaacgctagg  ggcgggatct  ggcggagtgg
61  aagaaccgcg  gcagggggcca  agcctcctca  actatgacct  caaccggcca  ggattccacc
121  acaaccaggc  agcgaagaag  taggcagaac  cccagtcgc  cccctcagga  ctccagtgtc
181  acttcgaagc  gaaatattaa  aaaggagcc  gtccccgct  ctatcccaa  tctagcggag
241  gtaagaaga  aaggcaaat  gaagaagctc  ggccaagcaa  tggaagaaga  cctaatacgtg
301  ggaactgcaag  ggatggatct  gaacctcgag  gctgaagcac  tggcttggtg  tggcttggtg
361  ttggatgagc  agttaaata  attccattgc  ctctgggatg  acagcttccc  ggaaggccct
421  gagcggctcc  atgccatcaa  ggagcaactg  atccaggagg  gcctcctaga  tcgctgcgtg
481  tcctttcagg  cccggtttgc  tgaaaaggaa  gagctgatgt  tggttcacag  cctagaatat
```

FIG. 6B-1

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541 attgacctga tggaaacaac ccagtacatg aatgaggagg aactccgtgt cctagcagac
601 acccacgact cagtttatct gcatccgaac tcatactcct gtgcctgcct ggccctcaggc
661 tctgtccctca ggctgggtgga tgcggctcctg ggggctgaga tccggaacgg catggccatc
721 attaggccctc ctggacatca cggccagcac agtccttatgg atggctatgg catgttcaac
781 cacgtggctg tggcagcccg ctatgctcaa cagaaacacc gcacccggag ggtcccttacc
841 gtagattggg atgtgcacca cgggtcaagg acacagttca cttcgacca ggaccccaagt
901 gtcctctatt tctccatcca ccgtacag cagggtaggt tctggcccca cctgaaggcc
961 tctaactggt ccaccacagg ttctggccaa ggccaaggat ataccatcaa tgtgccttgg
1021 aaccagggtg ggatgcggga tgcgtactac attgctgctt tcctgcagct cctgctgcca
1081 gtcgcccctc agctccagcc tcagctggct cagggtggcg ctggatttga tggcctgcaa
1141 ggggacccca agggcgagat ggccgccact ggccaggggt tggccagct aaccacctg
1201 ctcatgggtc tggcaggagg caagctgac ctgtctctgg aggtggcta caacctccg
1261 gccctggctg aaggcgtcag tgcttcgctc cacacccttc tgggagaccc ttgccccatg
1321 ccggagtcac ctggtgcccc ctgcccggag gccaggctt cagtttcctg tgctctggaa
1381 gcccttgagc ccttcttgga gttcttctg agatcaactg agaccgtgga gagggacaac
1441 atggaggagg acaatgtaga ggagagcgag gaggaaggac cctgggagcc cctgtgtctc
1501 ccaatcctga calggccagt gctacagtct cgcacagggc tggctctatga ccaaaatatg
1561 atgaatcact gcaacttgtg ggacagccac caccctgagg taccacagc catcttgcgg
1621 atcatgtgcc gtctggagg gctgggccct gccgggcgct gcctcacct gacaccgcgc
1681 cctgccacag aggtgagct gctcacctgt cacagtgcctg agtacgtggg tcatctccgg
1741 gccacagaga aaatgaaaac ccgggagctg caccgtgaga gttccaaact tgaactccatc
1801 tatactgcc ccagtacctt cgcctgtgca cagcttgcca ctggcgctgc ctgcccgcctg
1861 gtggaggctg tgctctcagg agaggtcctg aatgggtgctg ctgtgggtgcg tccccagga
1921 caccacgcag agcaggatgc agcttgccgt ttttgctttt tcaactctgt ggctgtggct
1981 gctcgccatg ccagactat cagtgggcat gccctacgga tcctgattgt ggattgggat
2041 gtccaccacg gtaatggaac tcagcacatg tttgaggatg acccagtggt gctatatgtg
2101 tccctgcacc gctatgatca tggcaccttc tccccatgg tcccatgagg tggcagcagc
2161 cagatcggcc gggccgcggg cacaggcttc accgtcaacg tggcatggaa cgggccccgc
2221 atgggtgatg ctgactacct agctgcctgg catcgccctg tgcttcccat tgcctacgag
2281 ttaaccacag aactggtgct ggtctcagct ggctttgatg ctgcacgggg ggatccgctg

FIG. 6B-2

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2341 gggggctgcc aggtgtcacc tgaggggttat gccacctca cccacctgct gatgggcctt
 2401 gccagtggcc gcattatcct taccctagag ggtggctata acctgacatc catctcagag
 2461 tccatggctg cctgcaactg ctccctcctt ggagaccac caccctgct gaccctgcca
 2521 cgccccccac tatcaggggc cctggcctca atcactgaga ccaccaagt ccatcgcaga
 2581 tactggcgca gcttacgggt catgaaggca gaagacagag aaggacctc cagtcttaag
 2641 ttggtcacca agaaggcacc ccaaccagcc aaacctaggt tagctgagcg gatgaccaca
 2701 cgagaaaaga aggttctgga agcaggcatg gggaaagtca cctcgcatc atttgggaa
 2761 gagtccactc caggccagac taactcagag acagctgtgg tggccctcac tcaggaccag
 2821 ccctcagagg cagccacagg gggagccact ctggcccaga ccatttctga ggcagccatt
 2881 gggggagcca tgctgggcca gaccacctca gaggaggctg tcgggggagc cactccggac
 2941 cagaccacct cagaggagac tgtgggagga gccatttctgg accagaccac ctcagaggat
 3001 gctgttgggg gagccacgct gggccagact acctcagagg aggtgtagg aggagctaca
 3061 ctggcccaga ccattctgga ggcagccatg gaggagcca cactggacca gactacgtca
 3121 gaggaggctc cagggggcac cgagctgac cgaactcctc tagcctcgag cacagaccac
 3181 cagacccccc caacctcacc tgtcaggga actacacccc agatatctcc cagtacactg
 3241 attgggagtc tcaggacctt ggagctaggc agcgaacctc agggggcctc agaattctcag
 3301 gccccaggag aggagaacct accaggagag gcagctggag gtcaggacat ggctgattcg
 3361 atgctgacgc agggatctag gggcctcact gatcaggcca tattttatgc tgtgacacca
 3421 ctgcccctgg gtcccattc ggtggcagta tgcccatac ctgcagcagg cctagacgtg
 3481 acccaacctt gtggggactg tggaacaatc caagagaact gggtgtgtct ctcttgctat
 3541 caggtctacc gtggtcgtta catcaatggc cacatgctcc aacaccatgg aaattctgga
 3601 cacccgctgg tcctcagcca catcgacctg tcagcctggc gttactactg tcaggccctat
 3661 gtccaccacc aggtctcctt agatgtgaag aacatcgccc accagaacaa gtttggggag
 3721 gatatgcccc accacacta agcccagaa tacggtccct ctacacctc tgagggcccac
 3781 gatagaccag ttccagcctg ttccaggctg taccttggat gaggggtagc ctcccactgc
 3841 atcccatcct gaatatcctt tgcaactccc caagagtgtt tatttaagtg ttaataacttt
 3901 taagagaaact gcgacgatta attgtggatc tcccctgcc catcgcccgc ttgaggggca
 3961 ccactactcc agcccagaag gaaagggggg cagctcagtg gcccacaagag ggagccgata
 4021 tcatgaggat aacattggcg ggaggggagt taactggcag gcatggcaag gttgcatacg
 4081 taataaagta caagctgtt (SEQ ID NO: 12)

FIG. 6B-3

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1 mdlrvqgrpp vepppeptll alqrpqrlhh hlflaglqqq rsvepmrlsm dtpmpelqvg
 61 pgeqelrqll hkdkskrsav assvvkqkla evilkkqaa lertvhpnsip gipyrtlepi
 121 etegatrsm1 ssflppvpsi psdppenhfpl rktvsepnlk lrykpksle rrknpllrke
 181 sappslrrrp aetlgdssps ssstpascgs spndsehgnp pilgdsdrrt hptlgprgpi
 241 lgsphtplf1 phglepeagg clpsrlqpil lldpsgshap lltvpglgpl pfhfaqsimt
 301 terlsqsglh wplsrttrsep lppsatappp ppgmqprleq lkthvqvikr sakpsekprl
 361 rqipseaedle tdgggpgqv ddglehrelg hqpeargpa plqqhpqvii weqqlagrl
 421 prgstgdcvi lplaqgghrp lsrqsspa pasisapepa sqarvlsse tpartlpflt
 481 gliydsvmk hqcscgdnsr hpehagriqs iwsrlqergl rsqceclgr kasieelqsv
 541 hserhvllyg tnplsrlkld ngklagilaq rmfemlpcgg vgvdtdtiwn elhssnaarw
 601 aagsvtdlaf kvasrelkng favvrppghh adhstamgfc ffnsvaiacr qlqqqskask
 661 askilivdwd vhhngtqqt fyqdpvlyi slhrhddgnf fpgsgavdev gagsgegfnv
 721 nvawaggldp pmgdpeylaa frivvmpiar efspdlvls agfdaaeghp aplggyhvsa
 781 kcfgymtqql mnlaggavvl alegghdlt a icdaseacva allgnrvdpl seegwkqkpq
 841 pqchplsgr dpgaq (SEQ ID NO:13)

FIG. 7A

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1 ataataccta ccttgacgga ccacgacagg attaagtgag gaaaaacccc catgagagt
61 ttttgccatt gtcaagttag cctgagggag gctgaggggg gatcaggctg tatcatgccc
121 ccgaggacaa actttccagt ttaccctgct cctctctctt gtccctaggc tgcccaggc
181 cctgcgcaga cacaccaggc cctcagccgc agcccatgga cctgcgggtg gcccagcggc
241 cccagtgga gcccaccaca gagccacat tgctggccct gcagcgtccc cagcgcctgc
301 accaccact ctctctagca ggcctgcagc agcagcgtc agtgaggctct
361 ccatggacac gccgacgccc gagttagcagg tgggacccca ggaacaagag ctgcggcagc
421 ttctccaaa ggacaagagc aagcgaagtg ctgtagccag cagcgtgttc aagcagaagc
481 tagcggaggt gattctgaaa aaacagcagg cggccctaga aagaacagtc catcccaaca
541 gccccggcat tccctacaga acccggagc cctggagac ggaaggagcc acccgctcca
601 tgctcagcag ccttcgcct cctgctccca gcccgccag tgaccccca gagcactccc
661 ctctgcgcaa gacagtctct gagcccaacc tgaagctgcg ccataagccc aagaagtccc
721 cggagcggag gaagaatcca ctgctccgaa aggagagtgc gccccccagc ccccgccggc
781 gggccgcaga gaccctcgga gactcctccc caagtagtag cagcacgccc gcatcaggg
841 gcagtcctcc caatgacagc gagcacggcc ccaatcccat cctgggcgac agtgaccgca
901 ggaccatcc gactctgggc cccgggggc caatcctggg gagccccac actccctct
961 tectgcccga tggcttggag ccgaggctg ggggcacctt gacctccgc ctgcagccca
1021 tctctctctt ggaccctca ggctctcatg ccccgctgct gactgtgccc gggcttggc
1081 ccttgccctt ccaactttgccc cagtccctaa tgaccaccga gcggctctct gggtcaggcc
1141 tccactggcc actgagccgg actcgctcag agccctgccc cccagtgccc accgctcccc
1201 caccgcccgg cccatgacg cccgcctgg agcagctcaa aactcacgtc caggtgatca
1261 agaggtcagc caagccgagt gagaagcccc ggctgcggca gataccctcg gctgaagacc
1321 tggagacaga tggcggggga ccgggcccagg tggtaggacga cggccccggag cacaggggagc

FIG. 7B-1

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1381 tgggccatgg gcagcccgag gccagaggcc ccgctcctct ccagcagcac cctcaggtgt
1441 tgctctggga acagcagcga ctggctgggc ggctcccccg ggcagcacc ggggacactg
1501 tgctgcttcc tctggcccag ggtgggcacc ggcctctgtc ccgggctcag tcttcccag
1561 ccgacactgc ctcaactgtca gcccagagc ctgccagcca ctgccagtc ctctccagct
1621 cagagacccc tgccaggacc tgcccttca ctgacctca ccacagggt gatctatgac tgggtcatgc
1681 tgaagcacca gtgctcctgc ggtgacaaca gcaggcaccc ggagcacgcc ggcgcacatcc
1741 agagcatctg gtcccggctg caggagcggg ggcctcggag ccagtgtgag tgtctccgag
1801 gccggaaggc ctccctggaa gagctgcagt cggctccactc tgagcggcac gtgctcctct
1861 acggcaccaa ccgctcagc cgcctcaaac tggacaacgg gaagctggca ggcctcctgg
1921 cacagcggat gttgagatg ctgccctgtg gtggggttgg ggtggacact gacacatct
1981 ggaatgagct tcattccLcc aatgcagccc gctgggcccgc tggcagtgctc actgacctcg
2041 ccttcaaatg ggcttctcgt gagctaaaga atggtttcgc tgtggtgcgg ccccaggac
2101 accatgcaga tcattcaaca gccatgggt tctgcttctt caactcagtg gccatcgct
2161 gccggcagct gcaacagcag agcaaggcca gcaaggccag caagatcctc attgtagact
2221 gggacgtgca ccatggcaac ggcacccagc aaaccttcta ccaagacccc agtgtgctct
2281 acatctccct gcctggccat gacgacggca acttcttccc ggggagtggg gctgtggatg
2341 aggtaggggc tggcagcggc gagggcttca atgtcaatgt ggcctgggct ggaggtcttg
2401 acccccccac gggggatcct ggtacactgg ctgctttcag gatagtcgtg acgcccacg
2461 ccgagagtt ctctccagac ctagtccctgg tgtctgcccgg atttgatgct gctgagggtc
2521 acccgcccc acLgggtggc taccatgttt ctgccaaatg ttttggatac atgacgcagc
2581 aactgatgaa cctggcagga ggcgcagtgg tgctggcctt ggaggggtggc catgacctca
2641 cagccatctg tgacgcctct gaggcctgtg tggctgctct tctgggtaac aggtgggac
2701 ccctttcaga agaaggctgg aaacagaaac cccaacctca atgacctcg ctctctggag
2761 gccgtgatcc ggggtgcacag taaatactgg ggctgcatgc agcgcctggc ctctgttcca
2821 gactcctggg tgcctagagt gccaggggct gacaaagaag aagtggaggc agtgaccgca
2881 ctggcgtccc tctctgtggg catcctggct gaagataggg cctcggagca gctgggtggag
2941 gaggaagaac ctatgaatct ctaaggctct ggaacctct gcccgccac catgccctg
3001 ggacctgggt ctcttctaac ccctggcaat agcccccat cctgggtctt tagagatcct
3061 gtgggcaagt agttggaacc agagaacagc ctgctgctt tgacagttat ccaggggagc
3121 gtgagaaaat c (SEQ ID NO:14)

FIG. 7B-2

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1 meepeepads gqslvpvviy speyvsmcde lakipkrasm vhsleayal hkqmrivkpk
 61 vasmeematf htdaylqhlq kvsqegdddh pdsieyglgy dcpategifd yaaaiggati
 121 taaqclidgm ckvainwsgg whhakkdeas gfcylndavl gilrlrrkfe rilyvdlldlh
 181 hgdgvedafs ftskvmvsl hkfspgffpg tgdvsdvglg kgryysvnpv iqdgigdeky
 241 yqicesvlke vyqafnpkav vlqlgadtia gdpmcdfnmt pvgigklky ilqwqlatli
 301 lggggynlan tarcwtyltg vilgkltisse ipdbefftay gpdyvleityp scrpdrneph
 361 riqqilnyik gnlkhvv (SEQ ID NO:15)

FIG. 8A

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1 gaaattcggc acgagctcgt gccgaattcg gcacgagaac ggttttaagc ggaagatgga
61 ggagccggag gaaccggcgg acagtgggca gtcgctggtc ccggtttata tctatagtcc
121 cgagtatgtc agtatgtgtg actccctggc caagatccc aaacgggcca gtatggtgca
181 ttctttgatt gaagcatatg cactgcataa gcaaatgagg atagttaagc ctaaagtggc
241 ctccatggag gagatggcca cttccacac tgatgcttat ctgcagcatc ccagaaggt
301 cagccaagag ggcgatgatg atcatccgga ctccatagaa tatgggctag gttatgactg
361 ccagccact gaaggatat ttgactatgc agcagctata ggaggggcta cgatcacagc
421 tgcccaatgc ctgattgacg gaatgtgcaa agtagcaatc aactggtctg gaggtggca
481 tcatgcaaaag aaagatgaag catctggttt tcgttatctc aatgatgctg tccctgggaat
541 attacgatgg cgacggaaat ttgagcgtat tccctacgtg gattcggatc tgcaccatgg
601 agatgggtga gaagacgcat tcagtttcac ctccaaagtc atgaccgtgt cctgacacaa
661 attctcccca ggatttttcc caggaacagg tgacgtgtcc gacgttggcc tagggaaagg
721 acggtactac agtgtaaatg tgcccatcca ggatggcata caagatgaaa aatattacca
781 gatctgcgaa agtgactata aggaagtata ccaagccttt aatcccaag cagtggctct
841 acagctggga gccgacacaa tagctgggga tcccatgtgc tcctttaaca tgactccagt
901 gggaattggc aagtgtctca agtacatccc tcaatggcag ttggcaacac tcatttcggg
961 aggaggaggc tataaccttg ccaacacggc tcgatgctgg acatactga ccggggtcat
1021 cctagggaaa acatatcct ctgagatccc agatcatgag tttttcacag catatgggtcc
1081 tgatttatgtg ctggaaatca cgccaagctg ccggccagac cgcaatgagc ccaccgaat
1141 ccaacaaatc ctcaactaca tcaaaaggaa tctgaagcat gtggtctagt tgacagaaag
1201 agatcagggt tccagagctg aggagtgggtg cctataatga agacagcgtg tttatgcaag
1261 cagtttgrgg aatttgtgac tgcagggaaa atttgaaaga aattacttcc tgaaaaattc
1321 caaggggcat caagtggcag ctggcttcct ggggtgaaga ggcaggcac ccagagtcc
1381 caactggacc taggggaaga aggagatarc ccacatttaa agttcttatt taaaaaaca
1441 cacacacaca aatgaaattt ttaatctttg aaaattattt ttaagcgaat tggggagggg
1501 agtattttaa tcatcttaaa tgaaacagat cagaagctgg atgagagcag tccaggtt
1561 gtagggcagg aggcagctga caggcagggn tngggcctcn ggaccancca ngtggagccc
1621 tgggagagan ggtactgac ngcagactgg gagg (SEQ ID NO:16)

FIG. 8B

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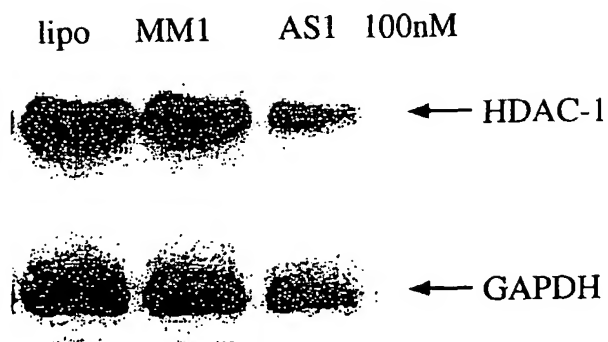


FIG. 9A

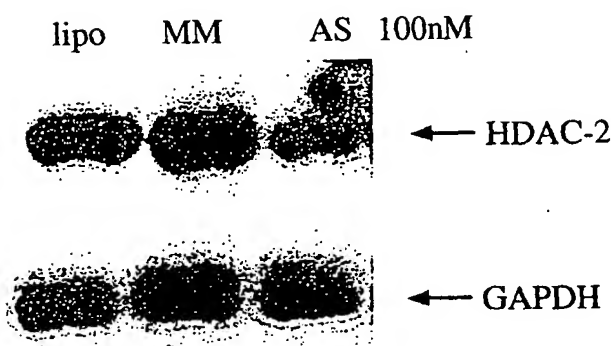


FIG. 9B

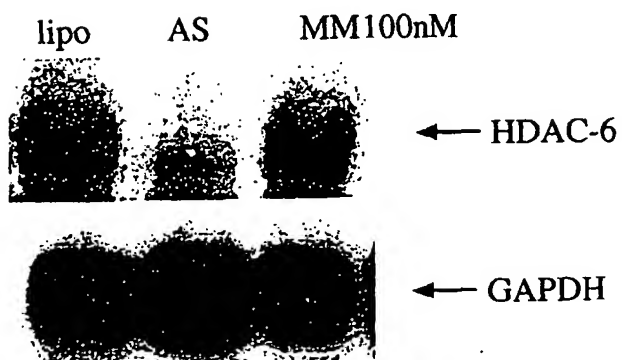


FIG. 9C

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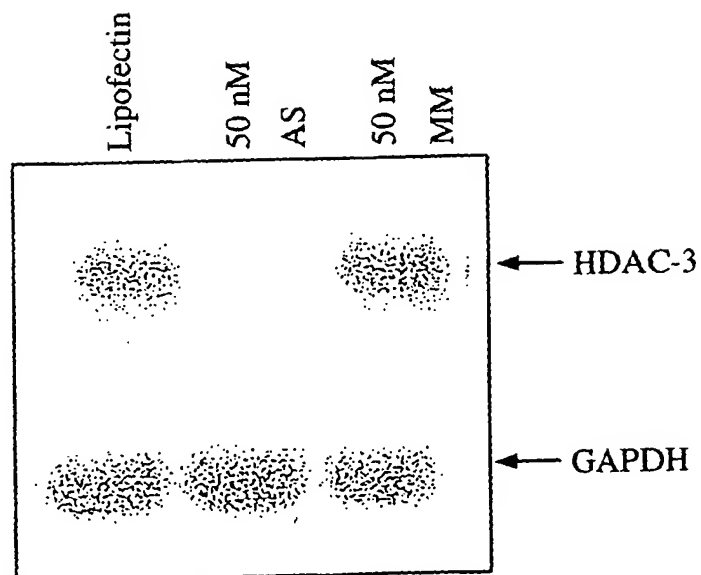


FIG. 9D

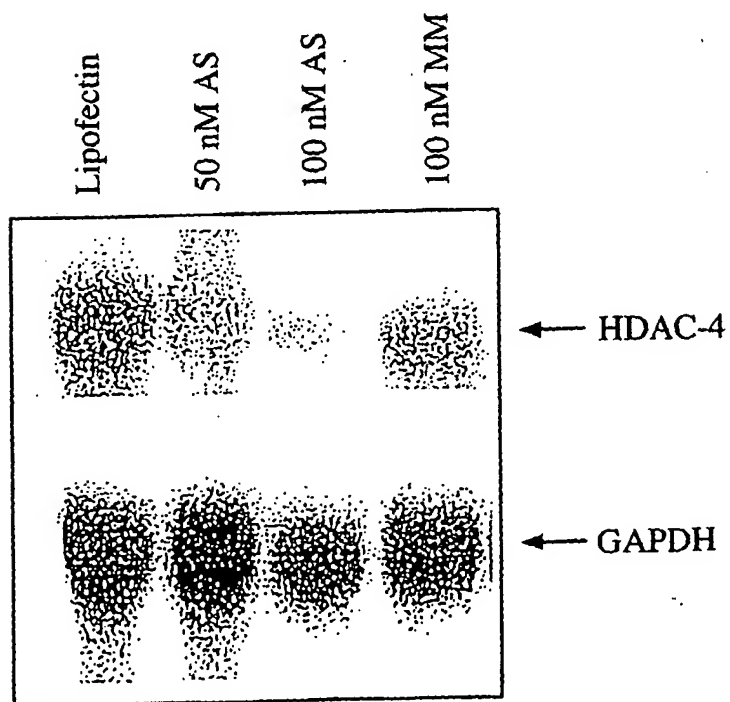


FIG. 9E

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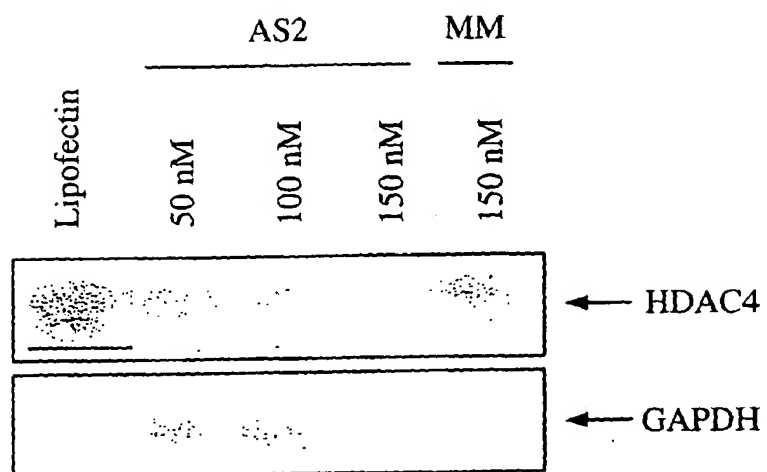


FIG. 9F

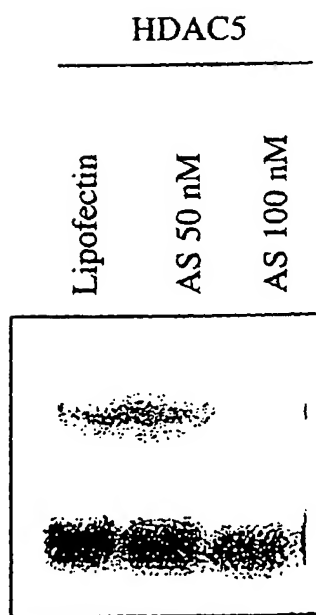


FIG. 9G

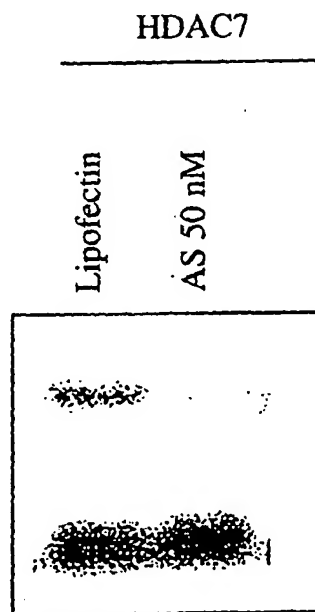


FIG. 9H

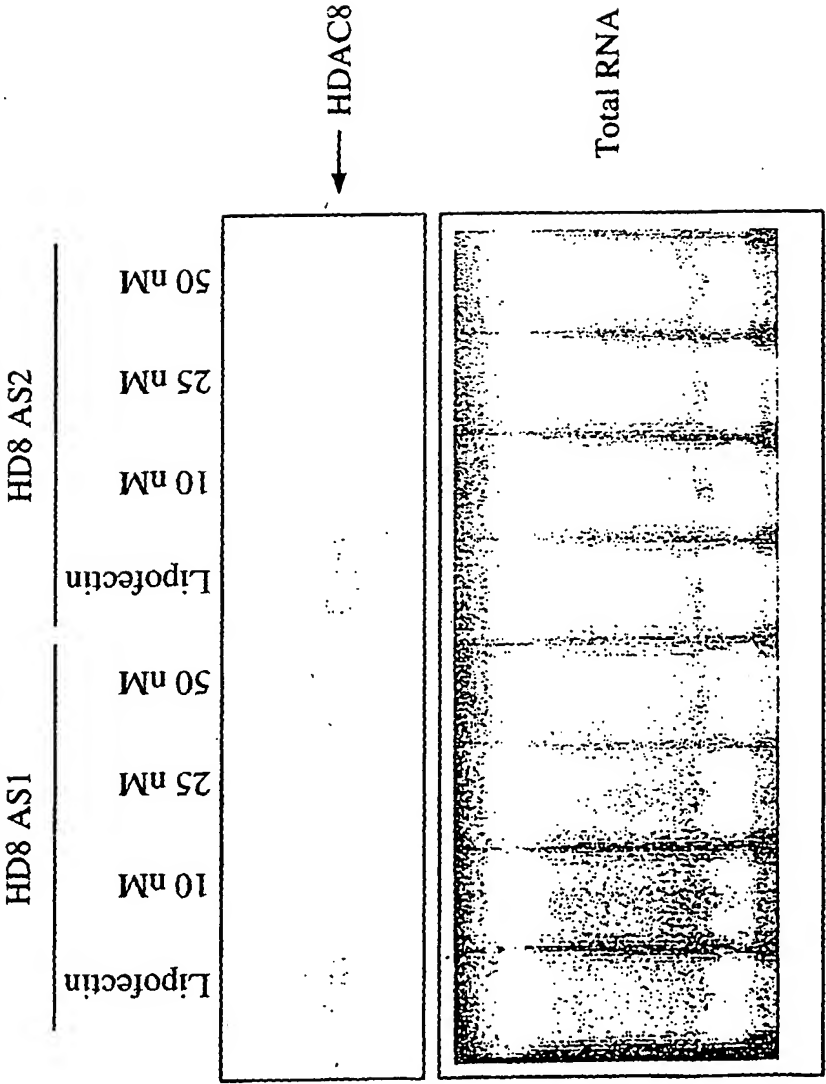
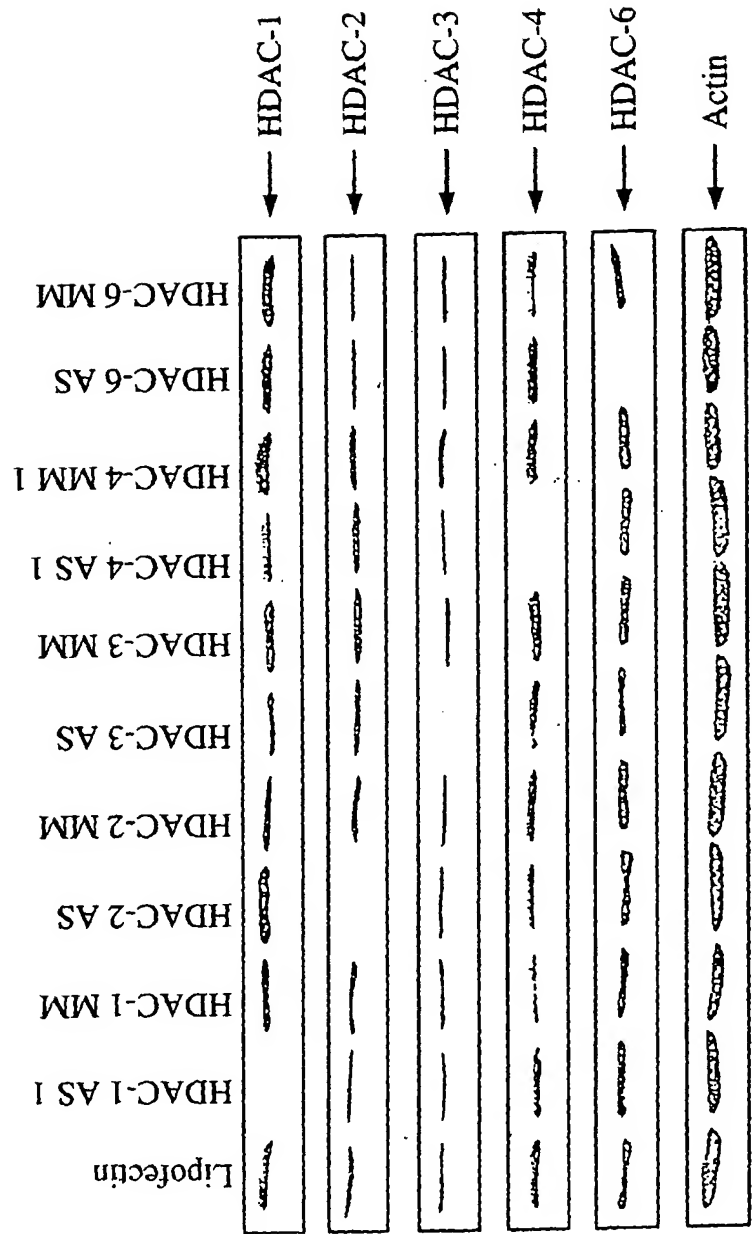


FIG. 9I



AS = Antisense
MM = Mismatch
NS = Non-specific control
3 day treatment
Oligonucleotide conc - 50nM

FIG. 10A

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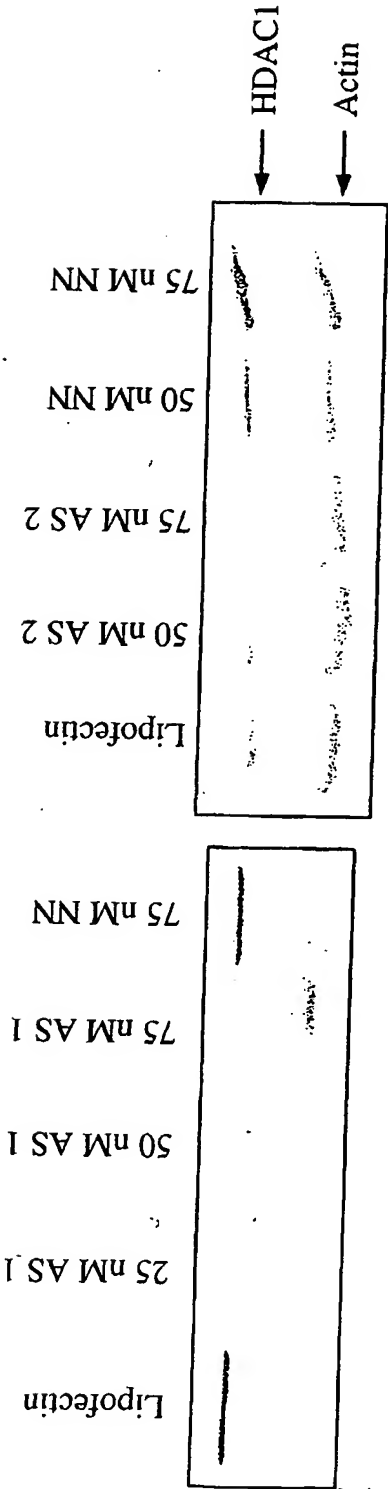


FIG. 10B

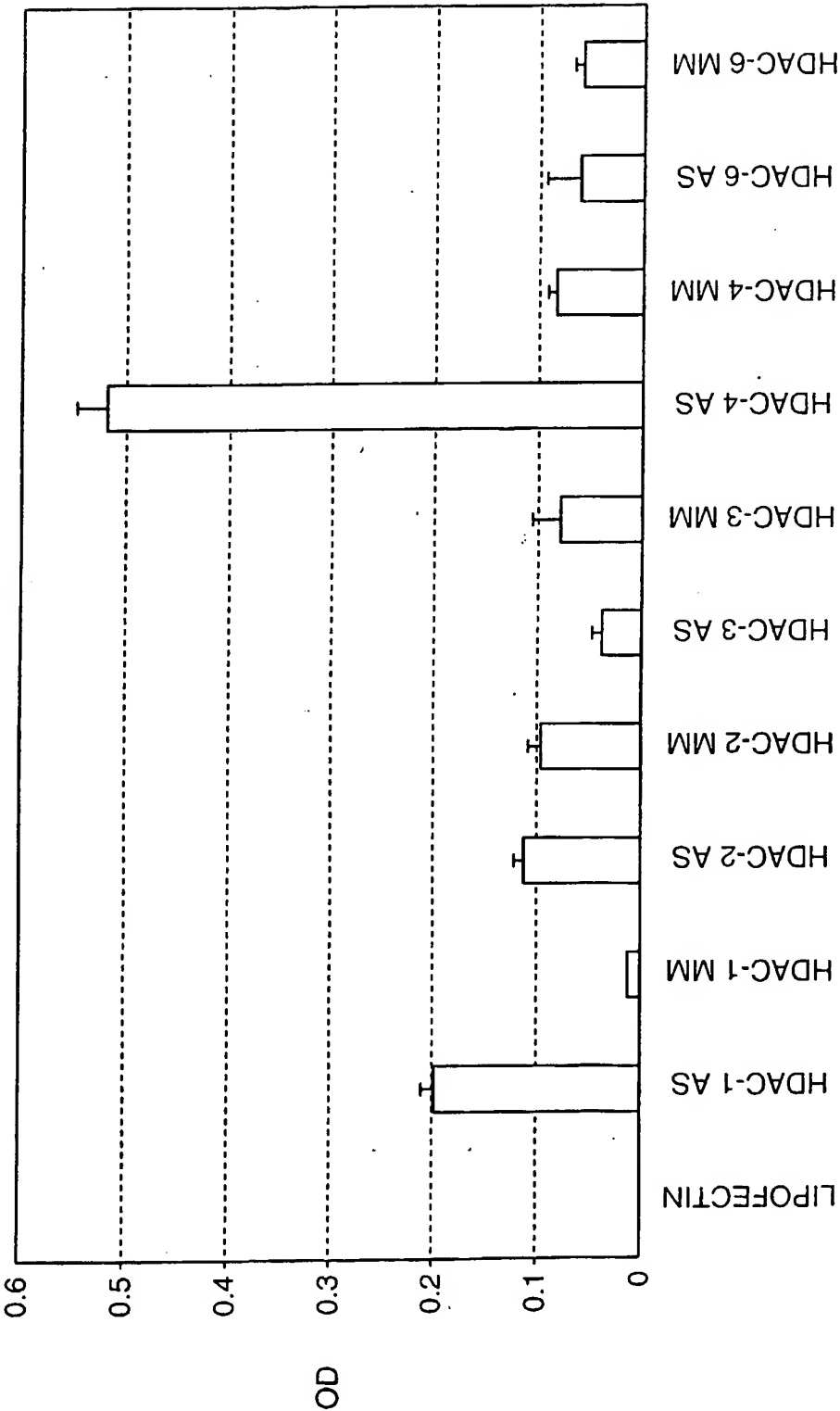


FIG. 11

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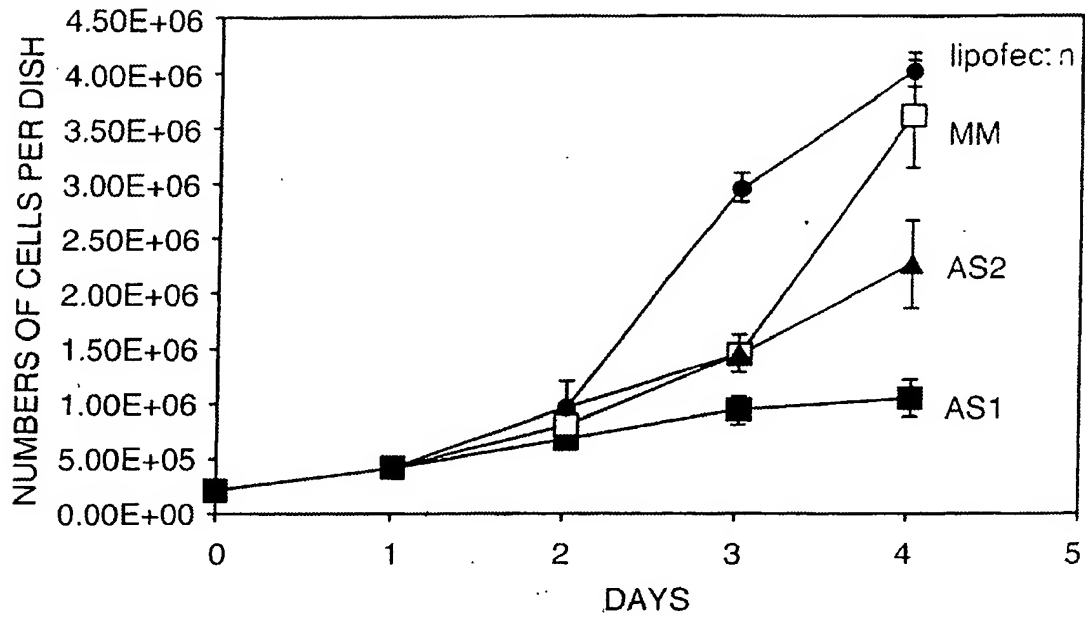


FIG. 12A

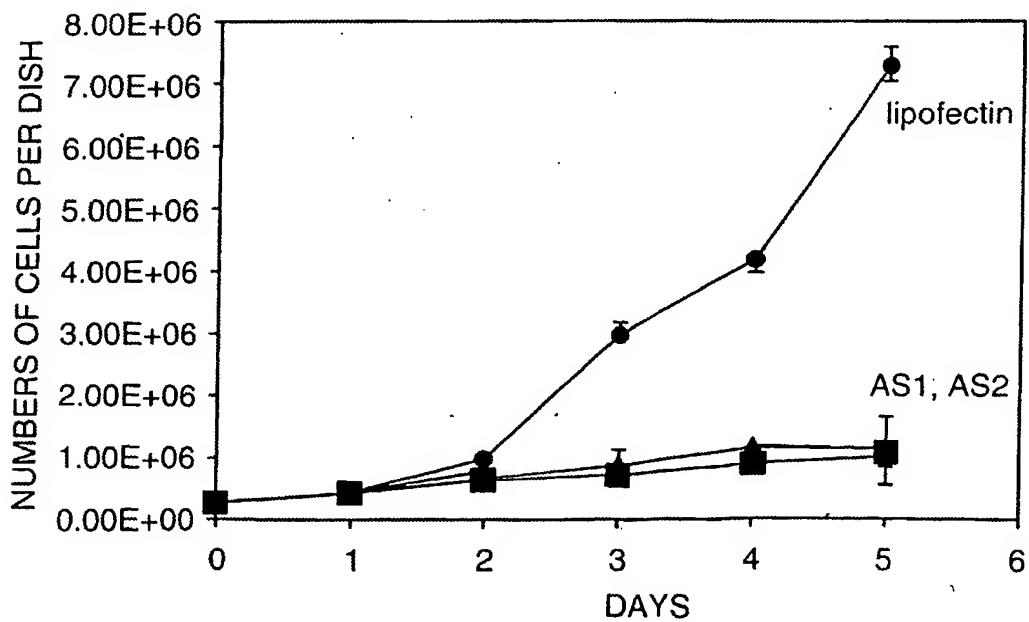


FIG. 12B

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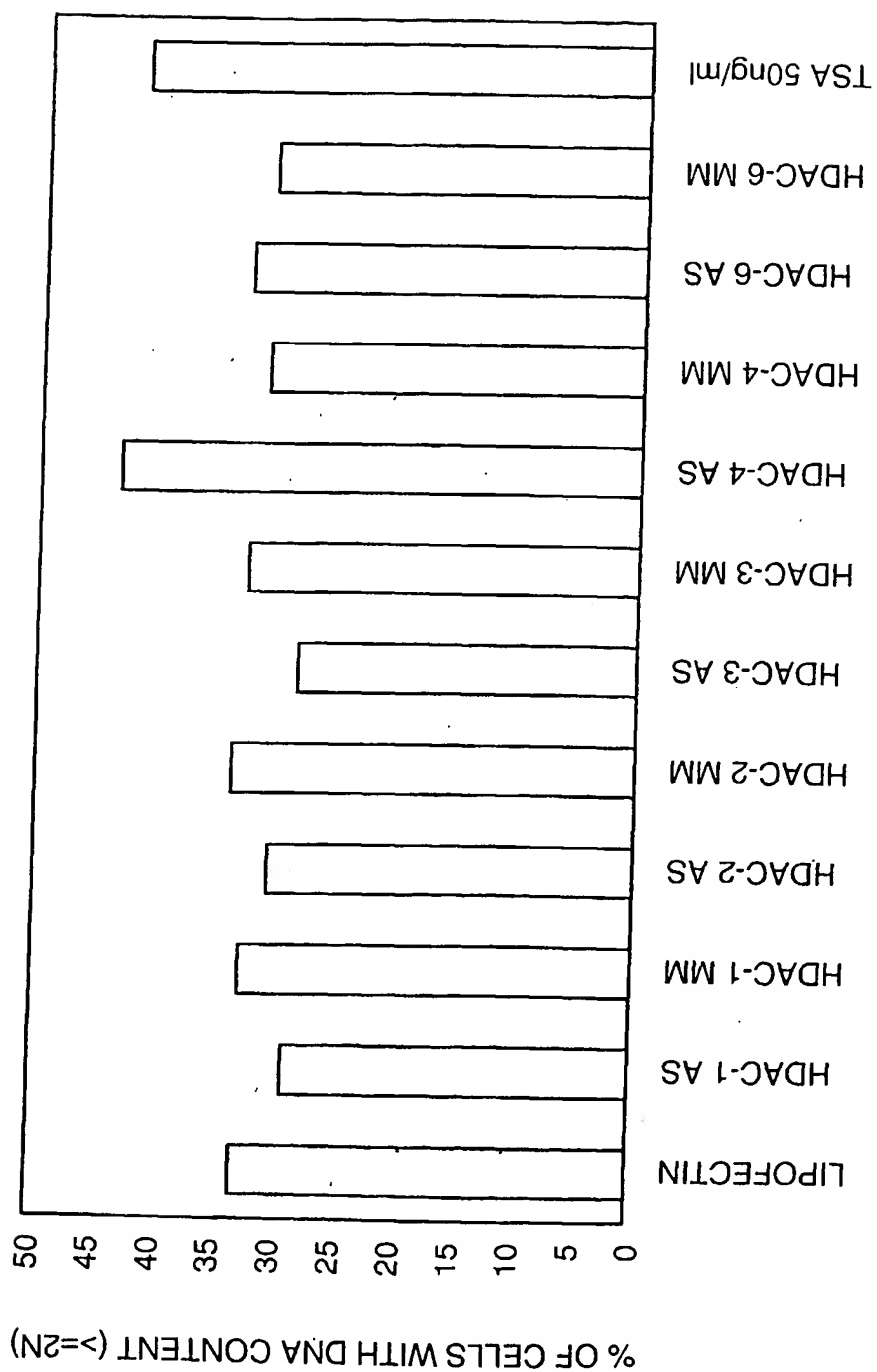


FIG. 13

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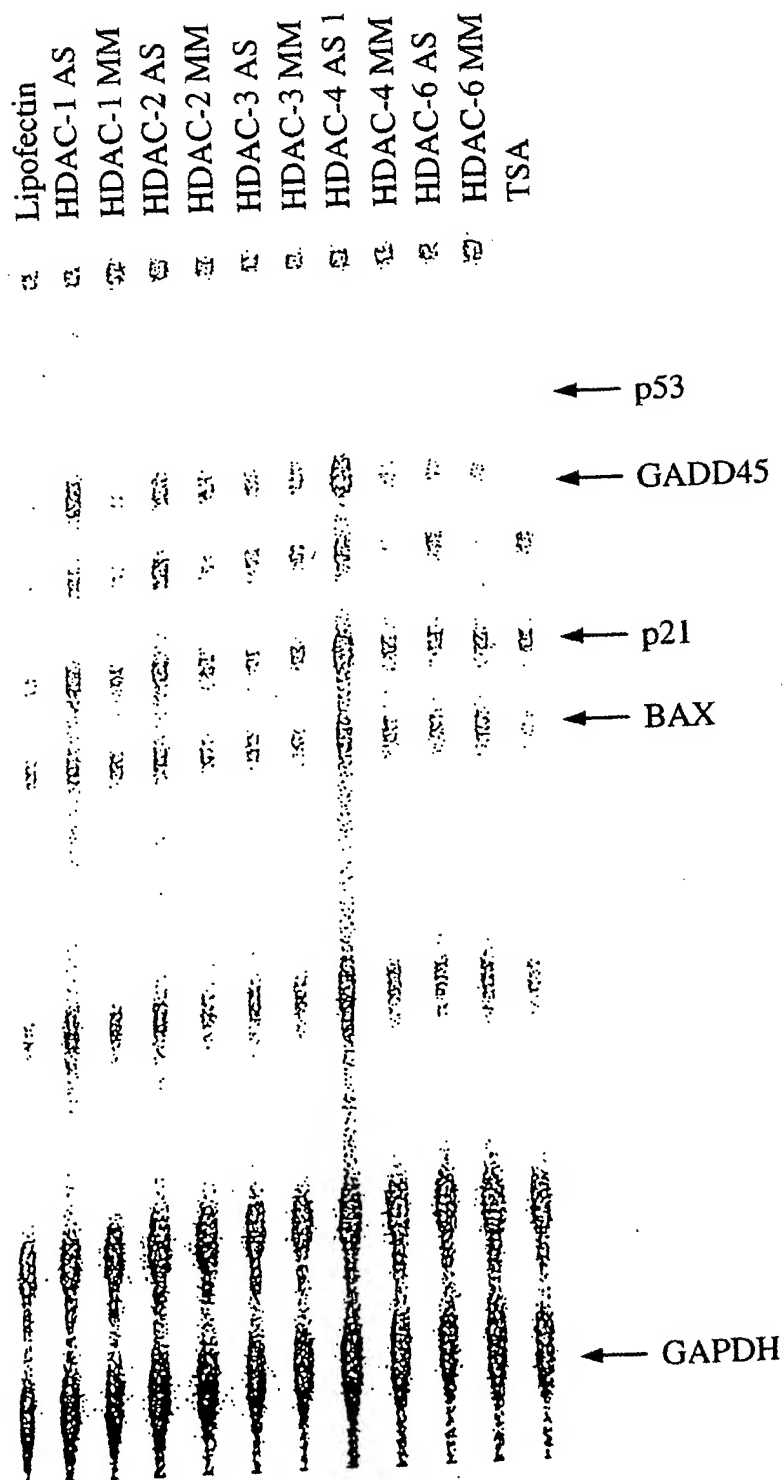


FIG. 14

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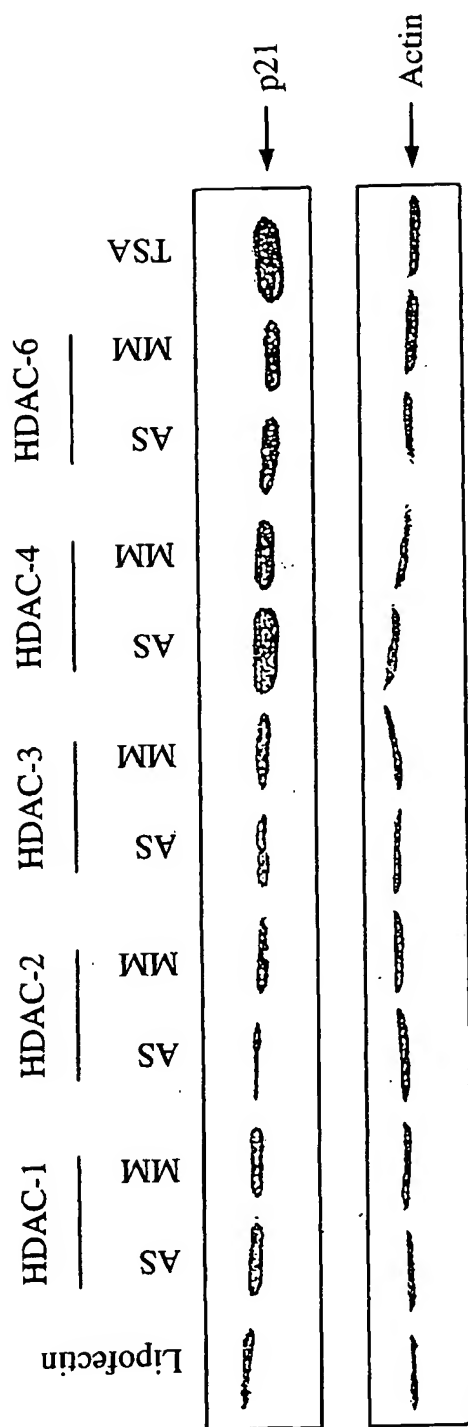


FIG. 15

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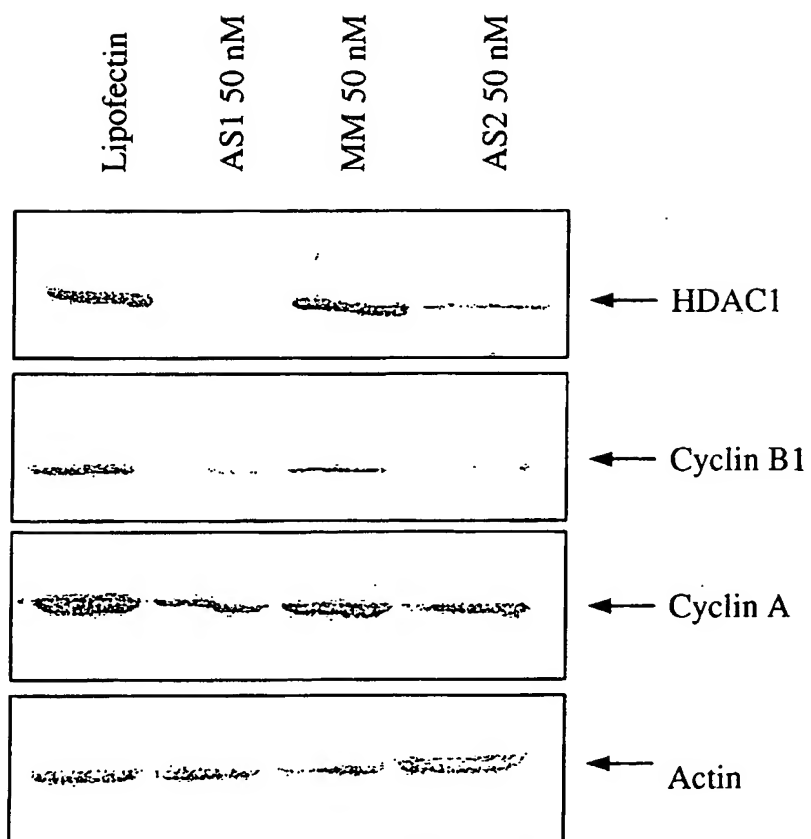
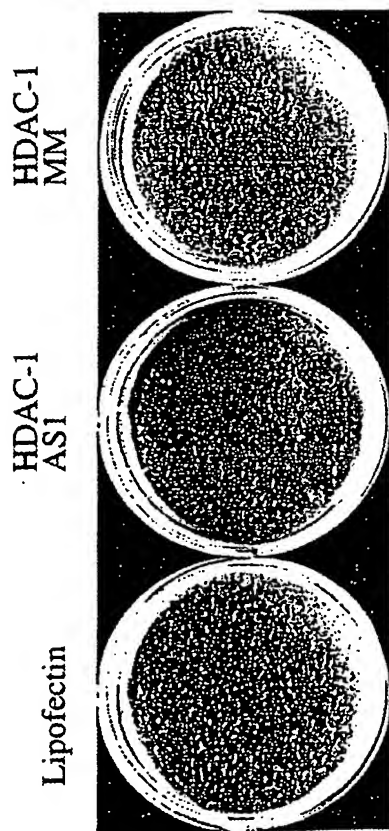


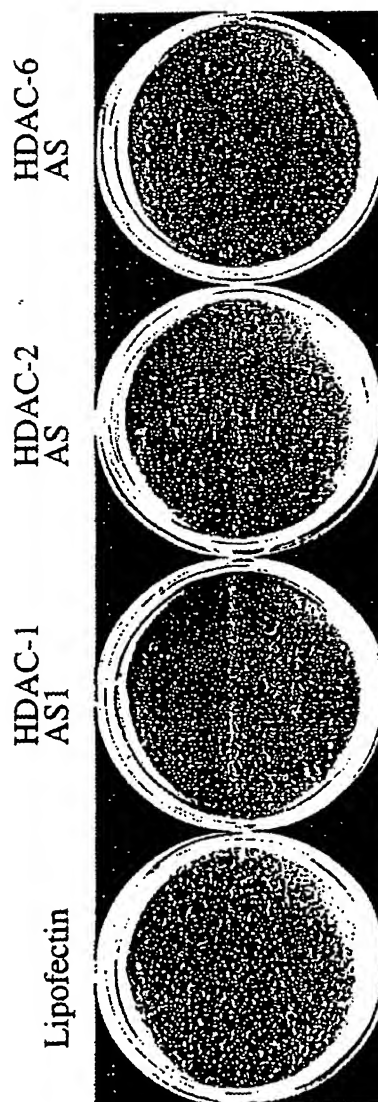
FIG. 16

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Colony Numbers -1200 -120 -1160

FIG. 17A



Colony Numbers -1200 -120 -890 -730

FIG. 17B

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